

**GENE EXPRESSION IN THE MICROFILARIAE
OF *BRUGIA PAHANGI***

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A thesis submitted for the degree of
Doctor of Philosophy

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June 2000

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Declaration

The studies described in this thesis were carried out in the Department of Veterinary Parasitology at the University of Glasgow Veterinary School between October 1996 and March 2000. The author was responsible for all the results except where it is stated otherwise.

No part of this thesis has been presented to this or any other University.

Richard D. Emes

June 2000

Acknowledgements.

I would like to thank my supervisor Eileen Devaney, for all the help and advice during my project. I am grateful to the MRC for funding this study and to Professor Andy Tait for the use of facilities in the Vet Parasitology department. Many thanks to Julie, Carol and Margaret for the assistance that keeps us all going, and to Colin Chapman for technical help that extended beyond the call of duty. Thank-you to Joyce Moore who helped to start this project and to Sarah Hunter for helpful advice. Many thanks to the various labs that provided parasite material for this project, Claude Maina (NEB), Jan Bradley (Salford University), William Harnett (Strathclyde University), Judi Allen and Laetitia LeGoff (University of Edinburgh) and Jean-Paul Akue (CIRMF, Gabon). Also to Mark Blaxter for the use of facilities in his lab in the University of Edinburgh, Xingxing Zang and David Guiliano for collaborating on various projects.

I also wish to thank all the members of the department, past and present especially those in the *Brugia* lab who have helped me in my endeavors and put up with my extended ranting. A special mention must go to Fiona Thompson, who has taught me so much molecular biology, runs an excellent taxi service and whose Granny's needlework skills have saved my sanity, my finances and my dignity respectively. Also to friends who have put up with my constant gibbering and complete lack of social skills whilst writing my thesis, especially Jeremy Kaye and Andy Bennett for helping to keep it all in perspective.

Thanks to Mum and Dad for encouraging me through life and for supporting me emotionally and financially for so long. Finally, of course the person who has helped me most and bared the brunt of my moans is Pauline, you were always there to put me back on track. In the words of '80s glum meisters The Mission, "You are my tower of strength".

Abbreviations.

bp	base pairs
BSA	bovine serum albumin
Ci	Curie
cm	centimetre
cpm	counts per minute
dNTP	deoxynucleotide triphosphate
ddH ₂ O	double distilled water
DNA	deoxyribonucleic acid
Dnase	deoxyribonuclease
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetic acid
ELISA	enzyme linked immunoabsorbent assay
EtBr	ethidium bromide
FCS	foetal calf serum
g	gram
gsp	gene specific primer
HBSS	Hanks balanced salt solution
IL	interleukin
kb	kilobase
kDa	kilodalton
λ	lambda
L1-L4	larval stage

M	Molar
mf	microfilariae
nt	nucleotide
PCR	polymerase chain reaction
p.i.	post infection
RNA	ribonucleic acid
RT-PCR	reverse transcriptase polymerase chain reaction
v/v	volume per volume
w/v	weight per volume

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Figure 5.35 Long-range PCR southern blot.

Figure 5.36 Long-range nested PCR of *B. pahangi* genomic DNA λ DASH II library to isolate the upstream region of *mmc-1*.

Chapter 6.

Figure 6.1 PCR of *mmc-1* ORF with *mmc-1*ExF1 and *mmc-1*ExR1 primers.

Figure 6.2 Induction of MBP-MMC-1 fusion protein expression in DH5 α competent *E. coli* cells.

Figure 6.3 Induction of MBP-MMC-1 fusion protein expression in Topp competent *E. coli* cells.

Figure 6.4 Pilot experiment of MBP-MMC-1 expression and purification.

Figure 6.5 Affinity purification of MBP-MMC-1 fusion protein.

Figure 6.6a Liberation of recombinant *mmc-1* by digestion with Factor Xa.

Figure 6.7 Testing the reactivity of MMC-1 antisera by Western blot.

Figure 6.8 SDS-PAGE of L3, mf and adult SDS protein extracts.

Figure 6.9 The recognition of MMC-1 in different life-cycle stages by Western blot.

Figure 6.10 Coomassie Blue stained *B. pahangi* mf E/S products.

Figure 6.11 Silver stained gel of *B. pahangi* mf E/S products.

Figure 6.12 Immunofluorescence analysis of MMC-1 localisation in sheathed and exsheathed mf of *B. pahangi*.

Figure 6.13 Immunofluorescent localisation of mmc-1 anti-sera in partially digested mf.

Figure 6.14 Effect of the inclusion of mmc-1 IgG in infected blood on the development of L3 stage parasites in the mosquito host.

Figure 6.15 Immunisation of mice with MMC-1 produces a specific IgG response.

Figure 6.16 Production of MMC-1 specific IgG in mf and L3 infections at 12 p.i.

Figure 6.17 IgG subclass recognition of mmc-1 in human *B. malayi* infections.

Figure 6.18a MMC-1 stimulated proliferative responses.

Figure 6.18b *B. pahangi* extract stimulated splenocyte proliferative responses.

Figure 6.19 Antigen specific production of IL-5.

Abstract.

Filarial nematodes have a complex developmental life cycle involving mammalian and mosquito hosts. The microfilariae (mf) which circulate in the blood stream are developmentally blocked prior to transfer to the mosquito. The switch between the different environments must be accompanied by changes in gene expression to allow development within the new host. The aim of this study was to isolate mf genes from the mf of *B. pahangi* that are differentially expressed within the two hosts.

To this end a mammalian-derived mf cDNA library was constructed and differentially screened using mammalian and mosquito-derived mf cDNA probes. Due to the difficulty of obtaining *in vivo* parasites from the mosquito, the cDNA probes were produced by RT-PCR from mf culture *in vitro* under either mammalian or mosquito like conditions.

Nine independent cDNA clones were isolated, of which five hybridised more strongly to the mammalian-derived mf probe and four to the vector-derived mf probe. Analysis of the cDNAs nucleotide sequence revealed that five of the clones were homologous to ribosomal protein mRNAs previously characterised from other species, one cDNA corresponded to the *B. pahangi* heat shock protein 90 mRNA and three cDNAs represented novel genes of unknown function.

A more detailed molecular analysis was conducted on two of the cDNAs of unknown function, Bp-vmc-2 (*B. pahangi*-vector-derived mf cDNA-2) and Bp-mm-1 (*B. pahangi*-mammalian-derived mf cDNA-1). Analysis of the expression of the mRNAs by semi-quantitative RT-PCR showed that Bp-vmc-2 was expressed at varying levels throughout the life-cycle of *B. pahangi*. The mRNA abundance of Bp-

vmc-2 increased as the parasite matured in the mosquito host and reached peak expression in infective L3 parasites. In contrast *Bp-mmc-1* was exclusive to the mf stage and was not expressed by mf developing *in utero*. An antiserum raised to the recombinant protein localised MMC-1 throughout the body of the mf, but it was not present on the surface of the sheath or cuticle of the parasite.

Western blotting using MMC-1 antiserum, reacted specifically with mf extracts, confirming the specificity of temporal expression. Analysis of immune responses to the recombinant protein showed that significant amounts of IL-5 were produced by T-cells in response to stimulation with MMC-1. Human serum from patients infected with *B. malayi* recognised MMC-1 and the predominant reactive immunoglobulin subclasses were IgG1 and IgG3, which have been associated with disease pathology in other studies.

CHAPTER ONE

1.1 The parasite.

1.1.1 Filarial nematodes.

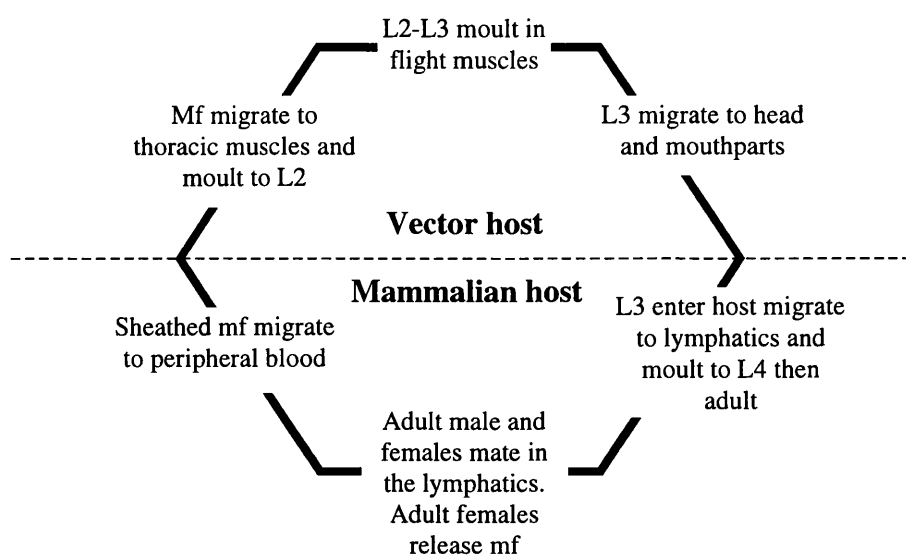
The filarial nematodes are blood and tissue dwelling parasites transmitted by the bite of a haematophagous arthropod vector. The parasites are accountable for a variety of diseases of both man and animals. In the case of human disease the most important species are the lymphatic dwelling filariae *Wuchereria bancrofti*, *Brugia malayi* and *Brugia timori* and the tissue dwelling *Onchocerca volvulus*, the causative agent of onchocerciasis or river blindness and onchocercal dermatitis. It has been estimated that one fifth of the worlds' population is at risk of lymphatic filarial infection [1] and over 128 million people are currently infected world-wide [2, 3]. Of these cases *W. bancrofti* is the most prevalent of the parasites (115.12 million infected) whilst Brugian filariasis accounts for 12.91 million cases [3]. The World Health Report (WHO) in 1995 named lymphatic filariasis as the second leading cause of permanent and long term disability world wide. Approximately half of the individuals infected with lymphatic filariasis have detectable morbidity, whilst the remainder show no overt clinical signs of infection [1]. The clinical manifestations of infection will be discussed later. These parasites are distributed through the tropical and sub-tropical regions of Asia, Africa the Western Pacific and parts of the Americas. The geographical distribution of the parasites is principally determined by the vector host. Bancroftian filariasis is very promiscuous in regards to vector and can be transmitted by various species of mosquito host. The most important of the vectors are *Culex pipiens* in urban areas and species of *Anopheles* and *Aedes* mosquitoes [4]. It is this diversity of vectors that promotes the vast distribution of bancroftian filariasis. Brugian filariasis is transmitted by mosquitoes of the genus

Anopheles, *Aedes* and *Mansonia*. *B. malayi* is the primary Brugian parasite of man but is also zoonotic and infects monkeys, various wild cats and the pangolin. Onchocerciasis is only transmitted by blackflies of the genus *Simulium*, primarily *S. damnosum* [4]. As the larval stage of the vector has an absolute requirement for fast flowing water, the distribution of the disease is localised to areas of flowing water. Onchocerciasis is mainly located in tropical and sub-tropical Africa. Lymphatic filariasis can occur in one of two forms, periodic where the mf display a periodicity in the peripheral blood to coincide with the feeding habits of the local vector, or sub-periodic, which does not show the periodicity of infection in the peripheral blood. *B. timori* discovered on the islands of Timor is a periodic parasite that exists in small foci in Indonesia. *B. pahangi* is a natural parasite of cats and dogs in Asia and it is an important laboratory model of Brugian filariasis as infections will develop to patency in a number of laboratory animals, including the jird *Meriones unguiculatus* [5], as will the sub-periodic form of *B. malayi* [6].

1.1.2 Life cycle.

The life cycle of Brugian nematodes follows the standard nematode plan of five developmental stages. Figure 1.1 shows a stylised diagram of the life cycle of the lymphatic dwelling filariae. Infection of the definitive host is initiated by the bite of a mosquito harbouring infective third stage larvae (L3) which are present in the head and mouth parts of the mosquito. During feeding the L3 are deposited onto the skin of the host. The L3 migrate through the puncture wound left by the mosquito and penetrate the dermis to reach the lymphatics. Within a few hours the larvae of *Brugia* have penetrated the local lymphatics and then flow to the perinodal sinus of the

Figure 1.1 Life-cycle of the lymphatic dwelling filariae.



nearest lymph node. The L3-L4 moult of *B. pahangi* occurs between days nine and twelve post-infection (p.i) [7], however in the gerbil model this moult occurs between days 6 and 7 p.i. The L4-adult moult is not synchronous, the males moult at around day 19 p.i. and the females later at around day 24. The juveniles then grow until sexually mature, usually between fifty and sixty days p.i. *B. pahangi* adult parasites have a thread-like appearance and measure approximately 0.1 X 22.0 mm (males) and 0.15 X 48.0 mm (females). The dioecious adults mate and the females continuously produce microfilariae (mf), the unique L1 larval stage characteristic of filarial nematodes. The mf of *B. pahangi* measure approximately 6 X 210 μm and are sheathed [7]. The mf migrate from the lymphatics to the circulatory system, from where they are ingested by a susceptible mosquito. In the vector the mf of *B. pahangi* migrate from the bloodmeal, penetrate the mosquito midgut and pass through the haemocoel before migrating to the site of development, primarily the thoracic muscles, where they initiate development [8]. Within 1 hour of feeding the mf can be detected in the flight muscles of a susceptible host [9]; the mf have lost their sheaths and lie in the muscle fibres and become sluggish. By 24 hours p.i. the mf have begun to shorten in length and increase in width (190 μm x 8 μm at 24 hours). By day three p.i. the mf is at its minimum length (172 μm x 16 μm) [9]. The mf are essentially motionless at this stage apart from infrequent twitching at the anterior end. During the late first stage, from the third to fourth day, numerous cell divisions can be observed as can a resulting increase in body length. The development of the parasite in the mosquito is temperature dependent; at 28 °C, 80 % humidity the parasites develop to the L2 stage between days 4 and 5, and by the eighth day the majority of the parasites are either L3 or in the process of moulting. The mature L3 then begin to

migrate to the head and mouthparts of the mosquito in preparation for the infection event. The development of the filarial nematodes is of interest as two defined stages of developmental arrest occur, at the mf and at the L3 stages. Both of these periods of arrest occur prior to transfer between hosts.

1.2 The human disease.

1.2.1 Clinical spectrum of disease.

Infection with *B. malayi* or *W. bancrofti* causes a wide spectrum of clinical manifestations which are summarised below.

Endemic normals

Members of this group display no symptoms of disease and are free of circulating mf despite residing in an endemic area. The diagnosis of an individual as an endemic normal is reliant upon the sensitivity of testing procedures. The most common test is to count mf in a specific volume of peripheral blood (see 1.2.2). Many cases that are believed to be endemic normals will harbour cryptic infections below the detection levels of the parasitological test or have pre-patent or single sex adult infections. With the advent of circulating antigen tests (see 1.2.2) that can reveal low level infections the percentage of true endemic normals has decreased.

Asymptomatic microfilaraemics

In endemic areas the majority of the infected population will be classed as asymptomatic microfilaraemics. These individuals show no overt symptoms of disease despite the presence of high levels of circulating mf. Although the patients do

not show clinical signs of infection many will have some form of subclinical disease. Approximately 40 % of cases have some renal damage that can be detected by urine examination [10], and many patients show a degree of lymphatic damage [11, 12]. The individuals in this group show a specific down-regulation of immune responses to filarial antigen and are often referred to as being hypo-responsive [13].

Filarial fevers

Filarial fevers are episodes of inflammation of lymph nodes accompanied by fever. The fevers occur periodically and may be associated with a loss of mf from the bloodstream. In immunological terms these patients are more responsive to filarial antigen and the clinical symptoms may be a result of immune reactions to parasites.

Lymphatic pathology

The term lymphatic pathology relates to damage in the lymphatic vessels which causes fibrosis and in severe cases results in blocking of the lymph vessels and disfiguring pathology. The symptoms are primarily caused by living and degenerating adult worms residing in the lymphatic vessels, although the conditions are often exacerbated by the presence of opportunistic bacterial or fungal infections [14]. Initially blocked or damaged lymphatic vessels result in lymphodema which is often treatable with chemotherapy. Following chronic lymphodema the lymphatics can become obstructed resulting in the debilitating chronic pathology conditions of elephantiasis and hydrocoele (in males infected with *W. bancrofti*). Patients with chronic pathology do not tend to have detectable mf in the bloodstream although a

small proportion of sufferers do exhibit circulating mf. The immune responses of individuals in this group show a recovered capacity to respond to filarial antigen.

Tropical pulmonary eosinophilia (TPE)

TPE is a rare condition of filarial infection which is thought to reflect an allergic response to mf in the lung [15]. In this condition mf are very rarely seen in the blood although treatment with DEC results in relief of symptoms. The wheezing cough and chest pain associated with TPE is thought to result from damage to the lung from degranulation of eosinophils in response to dead and degenerating mf present in the lung.

1.2.2 Diagnosis and treatment of lymphatic filariasis.

Diagnosis of lymphatic filariasis is classically by detection of circulating mf. This is achieved by collection of blood (at peak times in periodic species) by finger stick and generation of a thick blood film which is stained with Giemsa to highlight the mf. Alternatively a more sensitive method is to filter a larger volume of venous blood [16]. Generally 1 ml of blood is passed through a Nucleopore membrane with a 3 to 5 μm pore size. Retained mf can then be counted following staining with Giemsa. The disadvantage of this method is the need for venous puncture and also elevated cost. Adult worms can be visualised in the scrotal area of men suffering with *W. bancrofti* infections by utilising high frequency ultrasound which can detect motile adults within the dilated lymph vessels (termed the "filarial dance sign") [17]. As adult worms often remain in a constant location in so called "nests" this procedure

can be beneficial for monitoring the efficacy of drug therapy. The technique has also been attempted to detect *B. malayi* infections but with limited success [18].

Detection of circulating antigen is a beneficial means of diagnosis as the need for collection of blood at peak microfilaraemic times (often 10 pm-2 am) is avoided. The presence of filarial antigen in the blood of infected persons led to the development of immunological tests for detection of bancroftian filariasis. The tests use capture ELISA techniques which utilise monoclonal antibodies developed to circulating antigens of *W. bancrofti* [19, 20]. Unfortunately there is currently no such test for the diagnosis of Brugian filariasis. As an alternative to the detection of parasite antigen, which may be present in very low quantities in amicrofilaraemic individuals, molecular diagnosis utilises the PCR to amplify filarial-specific repetitive DNA sequences present in the blood or saliva of both *B. malayi* [21] and *W. bancrofti* infected individuals [22].

Serodiagnosis has been widely used to diagnose filarial infection by detection of filarial-specific antibody in patient serum. The techniques are based on ELISA tests that use parasite antigen as a means of detecting specific antibodies. As the antigens are prepared from worm extract, problems with standardisation of the procedure are inevitable. A potential problem with this technique is the cross reactivity of antibodies with antigens of other related helminths, particularly since many individuals in endemic areas have gastrointestinal nematode infections [23, 24]. Also sero-diagnosis does not distinguish between past and current infections. Another serological technique is the detection of anti-filarial IgG4 in patient serum [25]. IgG4 is the predominant antibody subclass observed in filarial infections [26].

This technique is useful as elevated IgG4 is only seen in current infections, although some degree of cross-reactivity between filarial species is observed.

1.2.3 Control of lymphatic filariasis.

Traditionally the treatment of filariasis was by a twelve day regime with the drug diethylcarbamazine (DEC). DEC primarily acts as a microfilaricide resulting in reduction of circulating mf for up to one year after chemotherapy. However the low compliance due to adverse side effects including headaches, vomiting and painful lymph nodes, has led to the more acceptable treatment of a single yearly dose of DEC (6 mg/kg) which has been shown to be as effective in reducing microfilaremia. DEC-medicated salt (0.2 % w/w) has also been used very successfully in areas where mass treatment is not feasible [27, 28]. The drugs Ivermectin (200 µg/kg) and Albendazole (400 mg) have also shown a potent effect against filarial worms. These drugs in combination with DEC or each other have been shown to yield 98 % and 99 % clearance of mf one year after treatment for *B. malayi* and *W. bancrofti* infections respectively [2, 29]. The advances in the effectiveness of these combination drugs and also the lack of a zoonotic reservoir for *W. bancrofti* and the fact that there is no multiplication of parasites in the vector host, has led an international task force on disease to name lymphatic filariasis as one of only six diseases that are potentially eradicable. The WHO initiative in collaboration with the healthcare companies SmithKline Beecham (donating albendazole) and Merck and Co (donating ivermectin) aims to eliminate lymphatic filariasis globally. Through the use of multi-drug therapy it is hoped to reduce the levels of microfilaremia so that transmission is no longer sustainable. The multi-drug therapy program is of added value as both

albendazole and ivermectin are effective against other helminth infections such as *Ascaris*, *Tricuris*, *Necator* and *Stronglyoides* which are often endemic in the same areas as lymphatic filariasis [30]. Also albendazole and ivermectin can safely be used in areas endemic for *O. volvulus* and *Loa loa*, where DEC cannot be administered due to the possible dangerous side effects such as ocular damage in *O. volvulus* infections [31], and encephalitis in *L. loa* infections [32].

1.3 The microfilariae.

1.3.1 Periodicity of the mf.

The mf of *Brugia* is a highly specialised stage evolved for life in the blood of the definitive host and for the transmission of the parasite to the mosquito. To increase the chances of transmission the mf of certain species have evolved a daily periodic life-cycle that maximises the mf count in the peripheral blood to coincide with the feeding habits of the local vectors [33-36]. This periodicity is also seen in a seasonal cycle that again mirrors the biting habits of the vector although this may be a reflection of alteration of the rate of mf production [37].

Experimental evidence on both *W. bancrofti* and *B. malayi* infected humans has shown that oxygen concentration of the blood is a determining factor in the periodic behaviour of mf. Inhalation of oxygen (13 minutes inhalation) during the night, when mf numbers are usually high, results in a 40 % reduction in the numbers of circulating mf [35]. In contrast body temperature [35] and modulation of the nervous system, by stimulation with acetylcholine [34] had no direct effect on the periodicity of *W. bancrofti* or *B. malayi*. These results led to the proposal that the mf were responding to the differences in the relative tensions of oxygen in venous and

arteriole blood. Generally during the day the mf accumulate in the arterioles of the lungs, where the oxygen tension is at its lowest before entering the pulmonary capillaries to become re-oxygenated [36]. It is thought that the mf leave the lung and circulate in the peripheral blood when the difference between O₂ tensions is less than 50 mm Hg and accumulate in the lungs when the difference is larger than 53 mm Hg, as would occur with activity during the day [35]. It has been postulated that the mf have evolved this process to avoid entering a zone of high O₂ tension known as the "oxygen barrier" and that different species of nematode may respond in a graduated fashion to display different types of periodicity [36]. Thus the nocturnal forms of *W. bancrofti* and *B. malayi* are proposed to be highly sensitive to the barrier and therefore only pass into the peripheral blood at night when the difference in venous and arteriole O₂ tension is low, whilst the mf of *Loa loa*, which are affected by temperature, are less sensitive to the barrier and will pass into the peripheral blood during the day [36].

How the mf respond to these physico-chemical cues is not understood, although it is known that the mf of *Brugia* parasites possess a differentiated nervous system and ciliated amphid sense organs [38]. It has also been shown that the mf of *B. malayi* express a novel gene for glia maturation factor (*Bm-gmf-1*) that promotes the differentiation of glia and neurons [39]. *Bm-gmf-1* is not expressed in adult female parasites but only in released mf suggesting that the neuronal system of the mf continues to develop when released from the adult worm. The mf of *Brugia* in the mammalian host were thought to be developmentally arrested and terminally differentiated meaning that no new structures were thought to be formed [38]. Due to the advances of molecular methods, which allow the detection of development not

apparent by anatomical studies this hypothesis may need re-evaluation. The development of the nervous system of mf may enable the mf to react to stimuli of the mammalian host such as those involved in the control of periodicity, or may allow the mf to respond to changes in physicochemical environment when transferred between the mammalian and mosquito hosts.

1.3.2 Non-continuous development of filarial nematodes.

As stated in section 1.1 the development of *Brugia* parasites is a complex process involving both mammalian and vector hosts. The development of the filarial nematodes is of interest as two defined stages of developmental arrest occur, at the mf and also the infective L3. Both of these periods of arrest occur prior to transfer between definitive and intermediate hosts. The mf can survive in the mammalian host in a developmentally arrested state for periods of greater than 100 days [40] and only re-initiate development when transferred to the vector. The mf are therefore the major reservoir of infection in endemic areas. The developmentally arrested mf of *Brugia* are still metabolically active as can be demonstrated by labelling of newly synthesised proteins [41]. The mf are therefore not a modified stage evolved to survive in harsh conditions, as is seen in some parasitic nematodes with a free-living stage [42, 43] or the specialised dauer stage of *C. elegans* [44].

Although the developmental block in *Brugia* is not analogous to the dauer larvae, the mechanisms by which the parasite assesses the environment may be similar. The dauer larval stage is an enduring stage that allows survival through periods of unfavourable conditions. The dauer stage is morphologically distinct, it is relatively thin due to radial shrinkage of the cuticle, the buccal cavity is sealed and

pharyngeal pumping is suppressed, resulting in resistance to desiccation and detergents [45]. The *C. elegans* dauer larvae also show decreased levels of transcription, (11-17 % RNA polymerase II transcription compared to other stages), and a reduced expression of a number of genes, collagen, actin, SL-1 and histone [46].

In *C. elegans* the developmental switch to the dauer pathway is made in response to environmental cues. Primarily the L1 are susceptible to the ratio of a dauer-inducing pheromone to food supply. The fatty acid pheromone, expressed by all life cycle stages of the nematode, promotes dauer formation and also inhibits the recovery from the arrested state, while the food signal acts in an antagonistic fashion [45, 47, 48]. The ratio of these two stimuli can therefore be used as a measure of the ability of the environment to support a given density of nematodes. When large numbers of nematodes are present and the food signal is low then the production of the dauer larvae is promoted to endure the period of environmental stress, whereas if food is abundant and can be exploited, then the dauer stage is not initiated.

It has been proposed that the free-living L3 stage of certain parasitic nematodes are dauer constitutive i.e. they must pass through a period of arrest comparable to the dauer [49]. Hotez *et al* (1993) documented the morphological and behavioural similarities between the L3 of hookworms and the *C. elegans* dauer, such as possession of a sealed buccal capsule and exhibition of questing behaviour. This article also proposed that the arrest of infective larvae is a result of evolutionary change that has removed the choice of developmental pathway. Therefore such nematodes possess a “dauer constitutive L3” to aid survival.

The control of dauer development is by the sensory organs (sensilla) that are present in concentric rings around the mouth [50, 51]. Two classes of chemosensory neurons termed ADF and ASI are involved in the process of dauer formation in *C. elegans* [47, 52]. The ADF and ASI neurons are pheromone sensors that repress dauer formation in response to absence of pheromone [47]. It has been found that homologous neurons control the developmental switching of the parasitic nematode *Strongyloides stercoralis* [52, 53]. The life cycle of *S. stercoralis*, like other parasitic nematodes of the genus *Strongyloides*, can develop along one of two routes in response to environmental stimuli. The soil dwelling larval stages can develop directly to infective stages (homogonic development) or develop to free-living adults (heterogonic development). By laser ablation studies it was shown that the ASF (homologous to *C. elegans* ADF) and ASI neurons which control *C. elegans* dauer formation are also responsible for the control of the developmental switch of *S. stercoralis* [52, 53]. It is therefore possible that the developmental block in the parasitic nematodes may be controlled by environmental stimuli which is monitored by similar neuronal structures.

Various studies have aimed to isolate genes that are involved in the maintenance of the developmental block in parasitic nematodes. Characterisation of the genes expressed in the arrested infective larval stage of *Toxocara canis* isolated a number of genes of interest [54, 55]. Of the abundant cDNAs sequenced from the arrested L3 stage of *T. canis* four novel transcripts were isolated (*ant*-003, *ant*-005, *ant*-030 and *ant*-035) which together represent 16.4% of the total library [54]. Although the function of these transcripts is unknown, their abundance suggest that they may be involved in the maintenance of the developmental block. Of the genes

isolated from this study of 261 ESTs it was noted that the level of expression of housekeeping genes was much reduced and no ESTs representing cell replication genes were isolated [55]. From this arrested stage library a prohibitin gene that is thought to play a role in suppression of cell proliferation was also isolated [56]. It must be noted that the prohibitin gene was isolated by PCR of the library and not by random sequencing of ESTs as described by Tetteh *et al* (1999)[54]. This suggests that the prohibitin gene is expressed at lower levels than the *ant* genes. However the finding of a gene involved in the control of the cell cycle is interesting as certain proteins may be involved in either the initiation or maintenance of arrested development. This phenomenon is also seen in the dauer larva of *C. elegans* where although the general levels of transcription are reduced, the level of the heat shock protein, *hsp90* was shown to increase in the dauer larvae [46]. The levels of *hsp90* mRNA were shown to be 10-15 times higher in the dauer than in other developmental stages, and reduced to approximately 5% of peak levels upon exit from dauer [46].

In *C. elegans* it is known that one of the dauer larva formation (*daf*) genes, *daf-7* encodes a member of the transforming growth factor- β (TGF- β) family [57]. Using fluorescent gene reporter studies (see section 1.4) it was shown that the *daf-7* gene was expressed in the ASI neurons in response to a food signal and that expression was regulated by the presence of dauer inducing pheromone. It was hypothesised that the *daf-7* gene is a negative regulator of dauer formation by transducing chemosensory information from the ASI neurons [57]. In attempts to identify genes involved in the developmental biology of filarial nematodes Gomez-Escobar *et al* (1998) used degenerate PCR to amplify TGF- β homologues of *B.*

malayi and *B. pahangi* [58]. The transcripts, *Bm-tgh-1* and *Bp-tgh-1* were isolated and showed higher homology to the *Drosophila* decapentaplegic protein (*dpp*) than to the *C. elegans* *daf-7* gene. The *dpp* gene is involved in the developmental patterning of the *Drosophila* embryo [59]. Expression of the *Bp-tgh-1* was low in L3 and absent in the mf, when the parasites are arrested, but expression is elevated at times coincident with the larval molts in the mammalian host [58]. It was hypothesised that *tgh-1* may play a role in the development of the parasite, and may influence the periods of developmental arrest [58]. Analysis of the expression of the *B. pahangi* TGF- β receptor (*Bp-trk-1*) showed that *Bp-trk-1* was expressed at high levels in the mf, when *Bp-tgh-1* is absent [58, 60]. It was therefore proposed that the expression of TGF- β following transfer of the mf to the mosquito may act as a trigger for the release of the developmental block [58].

A suggested hypothesis for the control of development was proposed by Petronijevic and Rogers (1983) [61]; in this model the different stages of development are controlled by sets of genes, some genes will be stage-specific whilst some will be present at various stages. The threshold for the switching of the genes is influenced by the environment, such that in a relatively constant environment the threshold for change is low and development will appear to be continuous. When development occurs in very different environments, as in *B. pahangi*, then the threshold is raised. Due to the higher threshold, development will only occur upon transfer to the new host, thus ensuring that development only proceeds in an appropriate environment [61]. In some parasites the stimulus for re-activation of development following arrest is known. For example in *Dictyocaulus viviparus*, a parasitic nematode of cattle, the presence of cattle bile stimulates the activation of

arrested larvae from pasture, which is likely to be related to the oral ingestion route of this parasite [62]. For an obligate parasite such as *Brugia*, the factors influencing the resumption of development are likely to be many-fold and result in a cascade of events that stimulates the expression of an appropriate gene set. Hawdon and Hotez (1996) proposed that when a parasite invades a host a "host-specific signal complex" is encountered which triggers this cascade of events [63].

The requirement of complex signals to initiate development of the mf of *Brugia* is evident in the fact that *in vitro* culture is complicated by the need for insect cells in cultures of mf to allow normal development to L2 stage parasites [64, 65], but despite this it is not possible to culture the parasites to L3. This is also evident in the complex culture systems required for other filarial parasites [66-72].

As discussed above there are examples of environmental cues influencing gene expression in a variety of nematodes. How are these signals received and relayed? Evidence for the transduction of an environmental signal to the interior of nematodes comes from the changes in surface coat following transfer of parasites from one environment to another. This was demonstrated by the association of lipid probes into the surface of various parasites (*B. pahangi*, *Acanthocheilonema viteae*, *Strongyloides ratti*, *Nippostrongylus brasiliensis*, *Trichinella spiralis* and *Ostertagia ostertagi*) exposed to mammalian conditions, that were occluded in non-mammalian conditions [73]. It was postulated that this change in surface properties could be a means of masking the surface antigens and evading the host immune response, or may be involved in a pathway of developmental signalling specific to the mammalian stage of the life cycle [73]. Subsequent studies showed that alkaline pH and Ca^{++} may stimulate these changes perhaps via a cyclic GMP, (cGMP) pathway [74]. In *T.*

spiralis incubation in culture medium containing the protease trypsin and/or bile causes the loss of the surface coat. This loss is believed to be activated through a Ca^{++} dependent messenger pathway acting via inositol triphosphate and cAMP [75]. The mechanisms of this pathway have been shown to be different in *T. spiralis* and *Brugia spp.* This may be due to taxonomic differences or the different routes of infection, via the gastrointestinal tract in *T. spiralis* and via the skin in *Brugia*. The role that the nervous system of the parasite plays in this cascade of events is unclear; the nervous system may be acted upon directly by these cascades although the use of nerve blockers did not interfere with the alteration of surface coat [74]. In *Brugia* the factors that may be involved in the triggering of a developmental switch are currently unresolved and are the subject of on-going research in this laboratory [41, 76-80].

1.3.3 The microfilarial sheath.

The mf of *Brugia* develop within the gravid female and are released as fully formed first stage larvae. The mf of the lymphatic dwelling filariae are enclosed in a bag-like structure known as the microfilarial sheath. The possession of the sheath is important as it represents the host-parasite interface of the blood-dwelling mf. The sheath is composed of two major components, a basal matrix layer which is a modified remnant of the egg shell, and an outer layer which is produced by the epithelium in the distal part of the uterus [81, 82].

Although the exact composition of the sheath is still unknown (due to the difficulty in obtaining sufficient amounts for standard analysis) it is known to contain a mix of proteins particularly rich in glutamine and proline [81] and has been proposed to contain chitin [82, 83], although this finding is not conclusive and it has

been disputed that chitin may only be present in pre-cursors of the sheath [81, 84, 85]. Components of the solubilised sheath can be separated to reveal 12-16 protein fractions from 14 to 200 kDa [81, 86]. A major sheath protein is a 22 kDa molecule, the mRNA for which has been shown to be transcribed in the oocytes and embryos of *L. sigmodontis* but not in the blood-dwelling mf [87-89]. The corresponding protein of *Brugia* is Mf22, which localised to the sheath of developing and mature mf by antibody staining [90]. However, northern blot analysis showed that expression of Mf22 was restricted to adult worms and not mf, suggesting that Mf22 is pre-synthesised by the adult female and later incorporated into the sheath [90].

As stated the sheath represents the surface that is exposed to the host immune system and therefore the sheath proteins are likely to represent important immunogens in Brugian infections. Serum from microfilaraemic *B. malayi* infected individuals has been shown to contain IgG reactive to sheath epitopes [91]. Also the appearance of antibodies to the mf sheath is known to correlate with the clearance of mf from the bloodstream both in animal models [92] and in humans [93].

1.3.4 Modulation of the host immune response by the mf.

The persistence of filarial infections in the lymphatics and blood stream suggests that evasion of the host immune response is a major factor influencing the interaction of parasite and host. Essentially only the factors that are thought to be relevant to the mf stage will be discussed here, although it is likely that the overall immune response is due to the presence of different parasite stages. Much research has been conducted on other aspects of immunology to filarial infections, for example [93-100]. As shown in section 1.2.1 the spectrum of clinical manifestations

resulting from filarial infections is very broad, ranging from the so-called endemic normals to gross pathology. It is generally considered that the interaction of the immune system and the parasite is correlated with disease. This is best exemplified in asymptomatic mf positive individuals where the immune system is hyporesponsive to filarial antigen, whereas chronic pathology sufferers show more aggressive immune responses and a lack of detectable parasites [13, 101-103]. A similar correlation between circulating mf and hyporesponsiveness has also been shown in animal models [92, 104] and in studies of human endemic communities [37, 93, 105, 106]. A direct role of the mf in this process was suggested by the studies of Mahanty *et al* (1996) who showed that the lack of proliferative response in *W. bancrofti* mf positive individuals compared to mf negative chronic pathology sufferers, was dependent on the source of antigen used in re-stimulation. Mf and mixed sex adult antigen (which contains large amounts of uterine mf) produced poor proliferative responses, whilst adult male antigen produced a good proliferative response [105]. In a further study on seasonal variation in mf levels, it was observed that unresponsiveness correlated to the presence of circulating mf during the seasonal periodicity of infection (see section 1.3.1) [37].

1.4 Cloning stage-specific genes from filarial nematodes.

Traditionally the isolation of genes from filarial nematodes has focused on the cloning of antigens by screening expression libraries with serum from infected individuals or animals. Although this approach has been successful in isolating genes of biological importance [107-109], the more direct approach of isolating genes that are temporally regulated or expressed at high levels has rapidly revolutionised

molecular parasitology. The approach of cloning genes that are temporally abundant is particularly useful as the genes that are up-regulated at specific time points are likely to be of importance in the biology of the organism being studied. The cloning of filarial genes is facilitated by the presence of the nematode SL1 22 nucleotide sequence at the 5' end of many mRNA transcripts. The SL1 sequence has been predicted to be present on > 70 % of *C. elegans* mRNAs [110] and on 80-90% of *Ascaris* mRNAs [111] and this is likely to be reflected in *Brugia spp.* The presence of the SL1 sequence allows a means to directly amplify first strand cDNA reverse transcribed with oligo (dT). This RT-PCR approach has been very successful in isolating abundantly expressed genes. Electrophoresis of amplified material produces a smear of products of varying sizes when visualised by ethidium bromide staining. Often intense bands representing abundant transcripts can be seen in the smear. These bands can then be extracted, cloned and analysed with relative ease. This type of analysis isolated many genes of potential importance in the biology of filariasis. By RT-PCR of mRNA from vector-derived L3, and cloning of a dominant amplified band Yenbutr and Scott (1995), characterised a *B. malayi* serpin (*Bm-serpin*) which is a potential immunomodulatory molecule present in the L3, adult and mf stages [112], and also a cuticular collagen, *Bm-col2* [113]. Gregory *et al* (1997) used this technique to isolate the abundant transcripts expressed by the vector-derived L3 (nine days p.i.) which included the abundant larval transcripts, *Bm-alt-1* and *Bm-alt-3*, and also the cystatin-type cysteine proteinase inhibitor *Bm-cpi-1* [114]. Analysis of the proteins encoded by these transcripts produced some interesting findings. *Bm-ALT-1* is a dominant antigen of L3 parasites and can act as a protective antigen against *B. malayi* in the gerbil model [98]. From these primary transcripts further members of the gene

family were identified by searching public databases. The *Bm-cpi-1* and the sibling member *Bm-cpi-2* are of interest as both proteins are thought to be located on the surface of the L3 once it has invaded the mammalian host. *Bm-cpi-2* encodes a protein that can inhibit the endopeptidases needed for antigen processing by B cells. Also if cultures of B and T cells are exposed to Bm-cpi-2 protein then no antigen processing occurs in the B cells and a subsequent decrease in T cell stimulation is observed (Rick Maizels, University of Edinburgh personal communication). If the Bm-cpi-2 protein is secreted from the L3 *in vivo* then this could be one of the factors involved in the down-regulation of the host proliferative response seen in filarial infections. Techniques that rely on cloning by such methods have also been successful in other parasite species, notably the nematode *Toxocara canis* [54, 115, 116], and *Ostertagia ostertagi* [117].

1.4.1 The filarial genome project.

Knowledge of the sequence of the complete genome of an organism, as has been achieved with various bacterial species and in the metazoan eukaryotes *C. elegans* [118], and more recently *Drosophila melanogaster* [119], provides a rich source of information regarding the biology of an organism. A catalogue of the genes present allows comparisons between organisms and identifies which genes are exclusive to any particular group. For a parasitic organism this information can reveal novel genes and metabolic pathways that are potential targets for chemotherapeutic intervention [120]. The filarial nematode *B. malayi* is one of several parasitic organisms being targeted in this way. The Filarial Genome Project (FGP) was initiated in 1994 through the World Health Organisation to conduct large scale

genomic investigation of *B. malayi*. Genomic sequencing on a scale needed for a nematode such as *B. malayi*, which has a haploid genome size of approximately 100 Mb [121], is beyond the scope of most research groups and so the initial aims of the FGP were to generate multiple cDNA libraries from parasites throughout the life cycle and to randomly sequence the 5' end of cloned cDNAs to produce expressed sequence tags (ESTs) corresponding to expressed genes [122, 123]. Although the EST programme is not complete it has compiled a prolific amount of data (20,773 ESTs in dbEST as of the 28th January 2000). The amount of information produced from sequencing projects can quickly become overwhelming. To make the information more accessible the FGP is part of the Parasite-Genome Resource Centre located at the European Bioinformatics Institute, Hinxton, UK, (www.ebi.ac.uk/parasites/parasite-genome.html). The website allows access to sequence information and home pages of different parasite projects and allows similarity searches by the BLAST algorithm [124] to genes of interest. To further aid researchers the ESTs isolated are grouped into "clusters" (correlating to predicted genes) and a consensus sequence of the predicted gene is stored (D. Guiliano and M. Blaxter, unpublished). By this method sequencing errors present in single ESTs are reduced and the frequency of isolation of the cDNAs from stage-specific libraries can be used to study the expression patterns of the genes throughout the life cycle. The advent of this type of information has allowed the practice of analysis "*in silico*" where the abundance and expression of genes of interest can be analysed by computer techniques [125, 126]. When the cluster database was searched to find clusters containing ESTs only found in the *B. malayi* mf stage library (SAW94LS-BmMF) 1165 clusters were isolated. To eliminate those genes that are simply

expressed at a low level and may not be truly mf-specific, the clusters that contained less than 3 ESTs were eliminated. 22 clusters (containing a total of 98 ESTs) were found to have 3 or more ESTs and are shown in Table 1.1. The majority of the mf-specific clusters contain genes that are currently of unknown function, some of which are expressed at high levels and may therefore represent genes of potential interest, e.g. the *amf* (abundant mf) genes. Amongst the genes with significant homology to genes of known function are structural proteins, phosphatidylinositol biosynthetic protein (*gpi-1*), and the housekeeping enzyme, aspartate aminotransferase (*aat-1*). The cluster that shows the largest number of ESTs is predicted to be the *B. malayi* homologue of cathepsin S (*Bm-cps*). This is a gene of interest as cathepsin S is a protease involved in antigen processing and presentation by MHC class II molecules. It has been shown that inhibition of cathepsin-S in dendritic cells leads to arrest of cells in an immature state and interrupts antigenic presentation [127]. To determine if *Bm-cps* could act as a true protease or could mimic true cathepsin-S function and perhaps disrupt the process of antigenic presentation would require experimental analysis of the corresponding protein. Characterised mf-specific cDNAs include the small heat shock gene of *B. pahangi* [80] which was found to be restricted to the mf stage under non-stress conditions and only expressed in the adult worms when exposed to high temperature stress (41°C, 2 hours). Another mf-specific gene is the serine protease inhibitor (serpin) (*Bm-spn-2*) which is very highly expressed in the mf library (49 isolated ESTs representing 2.1% of the total mf EST dataset) [128], although this gene is not present in the current edition of the cluster database and is therefore not represented in Table 1.1.

Table 1.1 *Brugia malayi* mf-library specific EST clusters.

Cluster	Number of ESTs	Gene name	Description
BMC00469	3	gpi-1	phosphatidylinositol biosynthetic protein.
BMC00480	3	BpcDNA5 protein	
BMC00630	3	not given	
BMC00685	3	wrr-1	similar to <i>Wucheria bancrofti</i> repeat region.
BMC00711	3	not given	
BMC00770	3	not given	
BMC00815	3	igl-1	contains immunoglobulin domain.
BMC01035	3	not given	
BMC01092	3	not given	
BMC01304	3	not given	
BMC01362	3	not given	
BMC01545	3	not given	
BMC06011	3	not given	
BMC00381	4	amf-4	
BMC00398	4	amf-3	
BMC00625	4	amf-2	
BMC01201	4	amf-1	
BMC01537	4	novel	
BMC06335	4	aat-1	aspartate aminotransferase.
BMC00312	8	amf-5	
BMC00546	12	wrr-2	similar to <i>Wucheria bancrofti</i> repeat region.
BMC00991	15	cps	cathepsin S

Table 1.1
The mf-library specific EST clusters that contain three or more individual ESTs.

The total number of ESTs contained in the mammalian-mf library is 2510 (beta release of the cluster database July 1999). Therefore the 98 mf-specific ESTs represent 3.90 % of the mf library. A similar analysis was conducted to compare the number of stage-specific ESTs from other life-cycle stages (Table 1.2). From this analysis it can be seen that the mf stage does not have a marked increase in stage-specific ESTs. The highest percentages of stage-specific ESTs observed are in the adult male and L3 libraries. It is interesting to note that the high number of ESTs that are specific to the L3 are from only a small number of clusters, i.e. the L3 may have a smaller number of stage-specific genes (clusters) but they are very highly expressed, e.g. the L3 specific clusters BMC00213 and BMC04934 which represent novel genes contain 86 and 28 ESTs respectively. This can be represented numerically if the number of ESTs are divided by the number of clusters that they are grouped within to give a mean number of ESTs per cluster. For the L3 this equals 31.8 ($159 \div 5$), much higher than for the other life cycle stages which are more comparable, mf = 4.45, L2 = 5.00, L4 = 7.50 adult female = 4.49 and adult male = 4.52. The high expression of these genes in the L3 may reflect the specialisation of this life cycle stage for initiation of infection in the mammalian host. The small number of stage-specific ESTs isolated from the L2 and L4 libraries probably reflects the smaller number of ESTs sequenced; initially the more abundant ESTs may be housekeeping genes found in all life-cycle stages and only after additional sequencing are the more lowly expressed ESTs that may represent stage-specific genes isolated.

In silico analysis has the reward of being able to quickly determine genes of potential interest. One gene isolated by this approach is the *B. malayi* macrophage migration inhibitory factor (*Bm-mif*, AS3ISB220) which was identified as a gene of

Table 1.2 Percentage of life-cycle specific ESTs isolated from *B. malayi* staged libraries.

Library.	Number of stage specific clusters in database. *	Total number of stage specific ESTs.	Total Number of ESTs in library. Ψ	Percentage of stage-specific ESTs.
Mf (SAW94LS-BmMF)	24	106	2510	4.22
L2 (JHU96SL-BmL2)	2	10	602	1.64
infective L3 (JHU93SL-BmL3 + SAW94WL-BmL3)	5	159	2622	6.06
L4 (JHU93SL-BmL4)	2	15	1060	1.42
Adult female (SAW94NL-BmAF)	21	103	3334	3.09
Adult male (SAW96MLW-BmAM)	42	190	2854	6.66

Table 1.2

Table of the percentage of stage-specific clusters of *B. malayi*.

* Number of clusters specific to a single life-cycle stage containing ≥ 3 ESTs

Ψ Number of ESTs sequenced and stored in β-1 release of FGP database July 1999.

potential interest from those expressed by *B. malayi* infective L3 [129]. The *Bm-mif* was the first demonstration of helminth production of a cytokine homologue that had the potential to modify the host immune response [130]. Following further analysis it was determined that the gene was transcribed in all stages but was elevated in the adult and mf stages of the parasite, and that the *Bm-MIF* protein was detectable in somatic extracts and the E/S products of cultured parasites [130]. Classically the MIF proteins are chemottractants for immune cells and therefore it may seem counter-intuitive for a parasite to attract effector cells, although it has been proposed that the stimulation of the host immune system in this way may promote parasite survival. It has also been shown that cytokines can be essential for the development and reproduction of helminth parasites [131].

The problems associated with relying on an *in silico* approach is that at present not all genes have been isolated. *B. malayi* has 20,000 predicted genes and the ESTs currently sequenced are thought to represent a total of 7,000 genes [121, 132]. As the sequencing effort is still continuing, the stage-specificity of a gene can only be predicted at present. It must also be considered that the stage-specificity of a gene is only based on the fact that a corresponding EST has not been isolated from another library. Therefore the quality of the program depends on the quality of the libraries that are produced. Thus some genes that are lowly expressed and may not be present in any library will be excluded.

1.4.2 Determining gene function in parasitic nematodes.

A major problem with the practice of *in silico* analysis is that although genes of interest can be selected, filarial worms are refractory to many forms of analysis

needed to determine gene function. One problem of filarial research is a long lived complex life-cycle, which in the case of *Brugia* requires both mammalian and vector hosts to allow normal development. In an attempt to overcome these problems many researchers have looked to model systems, especially the free-living nematode *C. elegans*. The comprehensive analysis of *C. elegans* which includes the full genome sequence, full cell lineage and extensive mapping of mutants provides a model organism in which the function of many genes can be predicted [133]. Where a *C. elegans* homologue of a gene of interest has been identified, extensive information is readily available and often a knock-out worm can be obtained for analysis. If this is not the case, a number of techniques are available using *C. elegans* as a heterologous transformation system. The conservation of regulatory elements between evolutionary divergent species can allow the analysis of spatial and temporal expression of a gene of interest. The promoter region of the gene of interest can be cloned in a reporter construct (*lacZ* or green fluorescent protein, GFP) and used to transform *C. elegans* by microinjection of the gonad and analysis of expression in progeny [134]. This approach was first used to study the benzimidazole resistant β -tubulin genes of *H. contortus* [135]. A similar technique has recently been used to analyse the expression patterns of the pepsinogen (*pep-1*) and cysteine protease (AC-2) genes from *H. contortus* and the cuticular collagen gene (*colost-1*) of *O. circumcincta* in *C. elegans* [136]. The results were shown to confirm the predicted spatial expression pattern (gut expression of AC-2 and *pep-1* and hypodermal expression of *colost-1*), although the temporal expression was not that predicted by RT-PCR analysis of the parasitic species [136]. This suggests that although the transcriptional machinery of the species tested is significantly homologous to allow

correct spatial expression, the elements controlling temporal expression are significantly variable. However, as yet there are too few examples to determine if this is a general effect. This type of transformation can also be achieved in *C. elegans* by ballistic delivery of particles coated with promoter elements of a gene of interest [137], and preliminary studies have shown that the transformation of parasitic species in this way may also be possible [138]. Ballistic transformation of *Ligmosoides sigmodontis* adult worms with the *actin-1* promoter resulted in the expression of *LacZ* reporter constructs that could be detected after culture of the parasite *in vitro* or in worms re-implanted in previously infected hosts [138]. Advances in the study of control of gene expression by these methods may overcome problems of working in a heterologous system. Other techniques available to attempt to determine gene function in *C. elegans* are microinjection of DNA from a gene of interest and assessment of the ability to rescue a specific mutant. Alternatively by cloning the open reading frame of the gene of interest downstream of a heat shock promoter, transformation of *C. elegans* and subsequent exposure to heat shock leads to over-expression equivalent to gain of function mutations [139].

Much of the analysis involved in determining the function of a cloned gene of interest relies on the availability of the correct model system. The advantages of working with a model organism such as *C. elegans* are considerable and for many genes this will provide valuable information about expression and function. However what degree of homology can be expected between parasitic nematodes and *C. elegans*? Blaxter *et al* (1998), showed by phylogenetic analysis of small subunit rRNA sequences that although the orders Rhabditida (*C. elegans*) and Strongylida (*Ostertagia ostertagi*, *Haemonchus contortus*) are closely grouped, the orders

Rhabditida and Spirurida (*Brugia spp*) are more distantly related [140]. However even though these nematodes are relatively close in evolutionary terms, the temporal pattern of expression of *C. elegans* genes under the control of promoters from *H. contortus* and *O. circumcincta* did not mirror what was predicted from RT-PCR analysis of the parasite species itself [136]. This may predict that more distantly related *Brugia* genes will prove to be more problematic to analyse in *C. elegans*.

Comparison of the ESTs isolated from *B. malayi* showed that 25 % of the ESTs are homologues of known genes from *C. elegans*, 17 % have homology to *C. elegans* genes of unknown function, 20 % have homology to known non-nematode genes and 38 % are unique to the filarial dataset. [132, 139]. From the *B. malayi* L3 stage, where the most complete analysis has been conducted 233 of 364 distinct genes (55 %) are predicted as *Brugia*-specific, and 10.4 % show homology only within the parasitic nematodes [129]. The large percentage of non-homologous genes is a challenge for parasite biologists as the parasite-specific genes are likely to encode proteins that are of most interest as potential targets of therapeutic intervention.

1.4.3 Cloning of differentially expressed genes.

Although utilising the Filarial Genome database would allow the prediction of mf-specific genes it would be very difficult to use this approach to isolate genes that are expressed differentially by a single stage in response to different host environments. Many techniques have been developed to allow the isolation of differentially expressed genes such as, differential display [141-143], differential library screening [79, 144-148], serial analysis of gene expression (SAGE) [149] and selective amplification via biotin- and restriction-mediated enrichment (SABRE)

[150]. For a general review of these techniques see [151]. Both SAGE and SABRE require a large commitment to the long term use of the techniques and are not practical for the small scale study planned here. Problems that have been encountered for the differential display technique are the levels of false positives that are produced [142] and the bias of the technique towards isolation of highly expressed mRNAs [152]. Therefore the method used to isolate genes in this study was a differential screen of a cDNA library. The differential screening technique is a method that allows the isolation of genes that are expressed at varying levels when exposed to different conditions [153]. The method used followed that described by Martin *et al* (1995) [154]. This method of screening an un-subtracted library with different populations of cDNA probe has a number of advantages. Primarily the technique is a very direct means of isolating products of interest, as a cDNA library is being screened the isolated product can be identified without further manipulation, as would need to be conducted with a genomic DNA library screen. Secondly the choice of material used for the production of the library influences the pattern of expression of genes isolated. In this case the screening of a mammalian-derived mf library enriches the library for transcripts that are expressed by the mf when in the mammalian host. In this study the gene expression of two distinct populations of parasite, microfilariae derived from mammalian and mosquito hosts, was compared.

This approach has been used previously in the laboratory to isolate genes that are up-regulated in mammalian-derived L3 [79], and also in vector-derived L3 [77]. These studies isolated genes that are potentially important in the initiation of infection of *B. pahangi* in the mammalian host. One such gene isolated from the 3 day post-infective L3 library was a cDNA (GenBank X91065) with homology to the

cytidine deaminase (CDD) gene family [79]. The CDDs are zinc-dependent enzymes which catalyse the deamination of cytidine nucleotides, a process known as RNA editing which can lead to the production of different protein products from a single mRNA transcript [155]. The *B. pahangi* CDD exhibits RNA binding but does not appear to edit the substrates tested [156]. This approach has also been used to isolate genes expressed in response to temperature changes encountered when transferring between hosts [80].

1.5 Aims of the project.

The aim of this project was to isolate genes that are differentially expressed in the microfilariae of *B. pahangi* when in the mammalian and mosquito hosts. As has been shown the mf are a highly evolved stage, modified for longevity in the mammalian host and with the ability to specifically down-regulate immune responses. It was anticipated that through the analysis of differentially expressed transcripts, genes that were involved in these phenomena may be isolated and their role in the biology of *B. pahangi* studied, in attempts to elucidate gene function.

CHAPTER TWO

2.1 Maintenance of parasite life cycle.

B. pahangi parasites were maintained by passage through a susceptible mosquito vector *Aedes aegypti*, strain (Ref^m) and mammalian host, the Mongolian jird, *Meriones unguiculatus*.

2.1.1 Maintaining susceptible mosquitoes.

Mosquitoes were housed in an insectary maintained at 28°C, with 70-80% relative humidity. Adults were confined in net cages, fed on 10 % sucrose and water from moist cotton wool pads. Larvae and pupae were held in plastic trays filled with tepid water and fed on yeast tablets. Pupae were isolated daily and placed in net cages in preparation for emergence as adults. Stock mosquitoes were starved for 24 hours and then fed on rabbit blood via a membrane feeding system (Haemotek) which maintains the blood at 37°C. Eggs were laid on moist filter paper and collected. The filter papers were dried and stored until required. To hatch the eggs, a filter paper was submerged in tepid water containing a yeast tablet.

2.1.2 Maintaining *B. pahangi* parasites.

Jirds were infected with 250 L3 in Hanks balanced salt solution (HBSS, GibcoBRL) by injection into the peritoneal cavity. After 3 months jirds were sacrificed by CO₂ anaesthesia and exsanguinated by cardiac puncture. Adults and mf were isolated from peritoneal washings with HBSS at 37°C. Adults were collected with a glass hook and transferred to a cryotube and stored in liquid nitrogen until further use. The mf were washed again with HBSS and then resuspended in rabbit blood at a density of 350-450 mf per 20 µl blood. Adult mosquitoes were fed for one

hour via the membrane feeding system following which the blood was replaced and the mosquitoes fed for a further hour.

2.1.3 Recovery of infective L3 stage *B. pahangi*.

Infective L3 were isolated from mosquitoes 9 days after infection, by the mass harvesting technique, [5]. The mosquitoes were collected from the net cages and placed in net bags. The mosquitoes were then crushed on a glass plate and washed with HBSS into a Baermann funnel lined with layers of gauze filled with HBSS and incubated at room temperature for 45 minutes. During the incubation the L3 emerge and collect at the bottom of the funnel. The L3 were washed at least twice in fresh HBSS. The L3 were then either used to infect jirds to continue the life cycle or frozen in liquid nitrogen until further use.

2.1.4 Recovery of mammalian-derived larvae.

750 vector-derived L3 in HBSS were used to infect jirds by intra-peritoneal infection. After the required period of development the jird was sacrificed and the parasites collected by peritoneal washing with HBSS. To collect the mf stage jirds infected with L3 at least 3 months previously were sacrificed and the mf collected from the peritoneal cavity by washing with HBSS. Contaminating red blood cells were lysed by the addition of 1 ml ddH₂O and the worms were then collected by centrifugation at 1000g for 5 minutes, and resuspended in 2 ml HBSS. The mf were then purified from any contaminating jird cells by centrifugation over a 5ml lymphoprep (Histopaque-1077 Sigma) gradient at 1200g for 15 minutes. Parasites

collected from the pellet were either used immediately or stored in liquid nitrogen until needed.

2.2 Molecular biology techniques.

Unless otherwise stated the chemicals used were AnalaR quality and were obtained from BDH chemicals U.K.

2.2.1 Standard PCR protocol.

PCR [157] was used to amplify DNA by a standard protocol.

Denaturation	94°C	1 minute.	}
Annealing	Primer Tm -5°C	1 minute.	} x n cycles
Extension	72°C	1 minute/kilobase.	}
Final extension	72°C	10 minutes	

2.2.2 "Hot Start" PCR.

The hot start protocol denatures the DNA template and primers prior to the addition of polymerase and dNTPs. This helps to reduce the chance of non-specific annealing and extension of non-specific sequences as the reaction heats from room temperature.

Reaction conditions for 100 µl reaction:

Denaturation:	94°C	3 minutes.	
	80°C	soak whilst PCR master mix of 1 X	
		polymerase buffer, 200 µM each dNTP,	
		2.5 units Taq polymerase	
		and 0.5 µM of each primer added.	
Denaturation:	94°C	1 minute.	}
Annealing:	Primer Tm -5°C	1 minute.	} x n cycles
Extension:	72°C	1 minute/kilobase.	}
Extension	72°C	10 minutes	

2.2.3 PCR mix for 100 µl reaction (Taq polymerase).

	per reaction	final concentration
10 X polymerase buffer	10 µl	10mM Tris-HCl,
(Perkin Elmer 100 mM Tris-HCl		pH8.3, 50 mM KCl,
pH8.3, 500 mM KCl, 15 mM MgCl ₂ ,		1.5mM MgCl ₂ .
0.01% w/v gelatin)		
dNTPs (10mM each, Perkin Elmer)	2 µl each	200µM each
Taq DNA polymerase (Perkin Elmer)	0.5 µl	2.5 units
Primer 1 (50 µM)	1 µl	0.5µM
Primer 2 (50 µM)	1 µl	0.5µM
Template DNA	n µl	
ddH ₂ O	to 100 µl	

2.2.4 Agarose gel electrophoresis

Typically agarose gels of 0.8%-2% (w/v) were used to separate DNA. The required concentration of agarose was mixed with 1 X TAE buffer and dissolved by boiling. The solution was allowed to cool for 10 minutes before adding 0.5 mg/ml ethidium bromide, except for gels used for genomic Southern blots. Gels were cast in GibcoBRL horizontal tanks using an appropriately sized comb to form wells. When set, sufficient 1 X TAE buffer was added to the tank to cover the gel. Aliquots of samples and DNA markers (usually *Hind* III or *Pst* I digested λ DNA) containing 1 % DNA loading dye were carefully loaded into the wells and allowed to settle.

For the electrophoresis of genomic DNA, gels were run at 25V and as the DNA migrated into the gel the voltage was then increased to 35V. For other DNA samples, the voltage was set at 50V and the DNA was run until an appropriate separation was achieved.

Reagents :

TAE buffer (50 X stock)	DNA loading dye (10 X)
2 M Tris base	25 % Ficoll (Sigma)
5.71 % (v/v) glacial acetic acid	0.4 % bromophenol blue
50 mM EDTA pH 8.0	0.4 % xylene cyanole FF
adjust to 1L with ddH2O	store at room temperature
store at room temperature	
Ethidium bromide stock (10mg/ml)	
One 100mg ethidium bromide tablet (Sigma) dissolved in 10 ml ddH2O	
store at room temperature in the dark.	

2.2.5 Preparation of restriction fragments for use as size markers in gel electrophoresis.

20 µg of λ DNA (GibcoBRL)

50 units of restriction enzyme

1 X React buffer

ddH₂O to 86 µl

DNA digested for 4 hours at 37 °C

add 4 µl 0.5 M EDTA and 10 µl 10 X DNA loading buffer = 0.2 µg/µl

2.2.6 Purification of DNA from agarose gels.

2.2.6.1 Purification by Spin-X columns.

0.8 % agarose gels were used for the purification of DNA, as the yield of DNA can decrease as the agarose concentration increases. The DNA was electrophoresed to separate the band of interest, visualised briefly on a UV trans-illuminator and the band carefully excised using a sterile scalpel. The agarose slice was transferred to a microcentrifuge tube and subjected to two rounds of freeze/thawing at -70°C for 15 minutes and at 60°C for 15 minutes. The contents of the tube were then transferred to a 0.22 µm cellulose acetate (Spin-X) column (CoStar) and TE buffer was added. The column was centrifuged at 13000g for 10 minutes, a further 200 µl TE buffer was added and the column centrifuged again. The collected DNA solution was precipitated by adding 40 µl of 3M sodium acetate and 880 µl of ethanol followed by incubation at -70°C for 30 minutes. The DNA was collected by centrifugation at 13000g for 15 minutes, salts were removed by washing with 1 ml 70% ethanol and the purified DNA was resuspended in an appropriate

volume of ddH₂O. The quantity of DNA recovered was assessed by running an aliquot alongside a known quantity of λ *Hind* III markers on a 0.8% agarose gel.

Reagents:

TE buffer (1 X)

10 mM Tris-HCl (pH8.0)

1 mM EDTA (pH8.0)

2.2.6.2 Purification of DNA from agarose gels by QIAquick gel extraction kit (Qiagen).

DNA was purified from 0.8 % agarose gels as per manufacturers' protocol and eluted into 30-50 μ l EB buffer (10mM Tris-HCl pH 8.5).

2.2.7 Ligation and transformation.

Fragments of DNA for cloning were either obtained by PCR with Taq polymerase and therefore the fragments had overhanging adenine nucleotides or from restriction digestion. DNA was ligated into either a TA vector (pCR2.1 Invitrogen) or pBluescript (Stratagene). The typical ligation reaction consisted of:

25-50 ng plasmid

10-50 ng DNA fragment

2 μ l of 5 X ligase buffer (GibcoBRL, final concentration 50 mM Tris-HCl (pH 7.6),

10 mM MgCl₂, 1 mM ATP, 1 mM DTT, 5 % (w/v) glycerol).

0.5 μ l (2 units) of T4 high concentration DNA ligase (GibcoBRL)

ddH₂O to 10 μ l

The reactions were incubated overnight at 16°C

Competent cells DH5 α (GibcoBRL), Topp cells (Stratagene), or One-shot INV α F' cells (Invitrogen) (for genotypes see 2.2.21) were transformed with an aliquot of the ligation reaction. The typical protocol used 20 μ l of DH5 α cells and 1 μ l of ligation reaction. The cells were defrosted on ice and the ligated plasmid added. The tube was incubated on ice for 30 minutes, followed by a heat shock at 42°C for 45 seconds. The cells were then placed back on ice for 2 minutes. Next, 200 μ l of SOC medium was added and the tube was incubated for 1 hour at 37°C, 225 rpm on horizontal shaker. The sample was spread over LB plates which contained 100 μ g/ml of ampicillin and the plates were incubated overnight at 37°C. For inserts cloned into plasmids where α -complementation allowed the determination of positive clones, the LB-amp plates were first coated with 50 μ l of 20mg/ml 5-bromo-4-chloro-3-indolyl-2-galactopyranoside (X-gal).

Reagents:

L-agar

7.5 g Bacto agar (Difco)
adjust to 500 ml with L-broth
dissolve and sterilise by autoclaving
cool to 45°C before pouring plates
(add antibiotics if required)
store agar plates at 4°C before use

L Broth

10 g Bactotryptone (Difco)
5 g yeast extract (Difco)
10 g NaCl
dissolve in 1L ddH₂O
aliquot into 100 ml bottle and autoclave
store at room temperature

Ampicillin stock (100 mg/ml)

1 g ampicillin (Sigma)

add sterile ddH₂O to 10 ml

store at -20°C

(use at 100 µg/ml in agar plates)

SOC Medium

20 g bacto tryptone

5 g yeast extract

0.5 g NaCl

25 mM KCl pH 7.0

add sterile ddH₂O to 1 L

sterilise by autoclaving

add 10 mM MgCl₂

and 20 mM glucose

X-gal 20mg/ml

Dissolve X-gal in dimethylformamide, wrap in aluminium foil and store at -20°C

2.2.8 In-gel Ligation.

To directly ligate DNA fragments separated, by electrophoresis, into a plasmid vector the fragment was first separated in a low melting point (LMP NuSieve agarose, FMC) agarose gel using low EDTA TAE buffer. The fragment was visualised and excised as for standard DNA fragment purification then melted by incubation at 65°C for 10 minutes. The DNA fragment was then diluted 1:10 with ddH₂O and ligated with 50 ng of appropriate vector using T4 DNA ligase as described in 2.2.7. The reactions were then incubated at room temperature overnight. Prior to transformation of the plasmid into a competent cell line the reactions were diluted 1:1 in ddH₂O after which the transformation protocol (as described in 2.2.7) was followed.

Reagents:**Low EDTA TAE (50 X stock)**

2 M Tris Base

5.71 % (v/v) glacial acetic acid

5 mM EDTA pH 8.0

adjust to 1 L with ddH₂O

store at room temperature

2.2.9 Plasmid miniprep.

This protocol is based on the alkaline lysis method for DNA purification [158]. Transformed bacteria were grown overnight in 3 ml of L broth (2.2.7) containing appropriate antibiotic, typically ampicillin (100 µg/ml). 1.5 ml of the culture was transferred to a 1.5 ml microfuge tube and the cells spun at 13000g for 1 minute and the supernatant discarded. The cells were resuspended in 200 µl of resuspension solution and mixed well. 200 µl of cell lysis solution was added, and the tube mixed to ensure total lysis, 200 µl of neutralisation solution was added and the sample was mixed again. The lysed cells were centrifuged at 13000g for 5 minutes to pellet the insoluble cellular debris, the supernatant was transferred to a clean microfuge tube and centrifuged for a further 15 minutes to ensure no debris remained. 500 µl of the supernatant was transferred to a fresh microfuge tube and 1 ml of ethanol was added the sample was mixed and incubated at room temperature for 10 minutes to precipitate the DNA. The sample was thoroughly mixed and then spun at 13000g to pellet the DNA. The supernatant was removed and the excess salt was removed by washing with 1 ml of 70 % ethanol. Following a further spin at 13000g, for 5 minutes, the DNA pellet was air dried and then resuspended in 30 µl of ddH₂O. The concentration of the plasmid solution was calculated by comparison of 1

μl of the preparation on a 1 % agarose gel alongside a known concentration of λ Hind III markers.

Reagents :

Cell resuspension solution

50 mM Tris-HCl pH 7.5, 10 mM EDTA, 100 μg/ml RNase A

Cell lysis solution

0.2 M NaOH, 1 % SDS

Neutralisation solution

1.32 M potassium acetate pH 4.8

2.2.10 Glycerol stocks of bacterial cultures.

0.5 ml of 50 % sterile glycerol added to 0.5 ml of bacterial culture and stored at -70°C.

2.2.11 Restriction digestion of DNA.

Plasmid and genomic DNA was digested at 37°C for between one and eighteen hours in an appropriate incubation buffer (React buffer GibcoBRL) with 1-10 units of enzyme (GibcoBRL).

2.2.12 Genomic DNA isolation

The procedure followed was modified from "A Protocol for Isolating High-Molecular-weight DNA from Mouse Tails" [159]. Approximately 300 mixed sex adult worms were transferred from liquid nitrogen to a 1.5 ml microcentrifuge tube containing 700 μl of genomic DNA extraction buffer and the worms were cut up using sterile stainless steel scissors. Next, 35 μl of 10mg/ml Proteinase K was added and the tube was incubated at 55°C overnight. 20 μl of 13 μg/ml RNase A (Sigma)

was added and incubated at 37°C for 2 hours. The tube was then filled with buffer-saturated phenol at pH 8 (GibcoBRL) and placed on a rotator for 1 hour. The two phases were separated by centrifugation at 13000g for 10 minutes. The aqueous phase (including the interface) was transferred to a fresh 1.5 ml tube. The tube was then filled with 1:1 phenol/chloroform (v/v), the contents were mixed by inversion and the phases were separated as above. The aqueous phase (including the interface) was transferred to a fresh tube, and the tube was filled with 24:1 chloroform/isoamyl alcohol. The contents of the tube were mixed by inversion and the phases were separated by centrifugation. The aqueous phase was transferred to a fresh tube (avoiding the interphase) and this tube was filled with isopropanol. The end of a thin glass capillary tube was sealed in a Bunsen flame and allowed to cool. The capillary tube was dipped into the isopropanol solution and the precipitated DNA was coiled around the glass by gently stirring the solution. The DNA (attached to the glass tube) was dipped into 70 % ethanol, then into 100% ethanol and allowed to air dry. A diamond-tipped cutter was used to score the capillary tube and the end (plus DNA) was dropped into a 1.5 ml microcentrifuge tube containing 500 µl of TE buffer pH 7.4. The tube was then placed on a rotator at 30-60rpm overnight and the glass tip was removed using sterile forceps. An aliquot of DNA was diluted 1/100 and used to assess the purity of the DNA by calculating the ratio of absorbance of DNA (260 nm) to protein (280 nm), which should be between 1.8 and 2.0. The concentration of the genomic DNA was calculated by the formula: (absorbance at 260nm) x 50 x (dilution factor) = mg/ml of DNA.

Reagents :**Genomic extraction buffer.**

50 mM Tris-HCl pH 8, 100 mM EDTA, 100 mM NaCl, 1 % SDS

TE buffer.

10 mM Tris-HCl pH 7.4, 1 mM EDTA

2.2.13 Preparation of genomic DNA for Southern blotting.

For a genomic Southern blot, 20µg of high molecular weight genomic DNA was digested with 10 units of an appropriate restriction endonuclease. The restriction digests were incubated in a volume of 400 µl at 37°C overnight and were precipitated at -20°C with 3 M sodium acetate pH 5.2 (40 µl) and ethanol (880 µl). The DNA was collected by centrifugation at 13000g for 20 minutes, the salts were removed with a 70 % ethanol wash (1 ml) and the DNA was resuspended at 37°C, in 30 µl ddH₂O for several hours. 10 % (v/v) DNA loading buffer was added to each sample and the fragments were separated on a 0.8 % agarose gel (TAE). DNA markers, were also added to allow size determination. After the bromophenol dye front had migrated 2/3 the length of the gel, the gel was stained for 30 minutes in ddH₂O containing 1.5 mg/ml ethidium bromide, viewed on a UV trans-illuminator and photographed along side a ruler. Gels were depurinated in 0.25 M HCl for 30 minutes to improve transfer of high molecular weight fragments. They were then soaked in denaturing solution for 30 minutes and in neutralising solution for 30 minutes. The gels were rinsed in ddH₂O between each solution. For PCR blots, the PCR products and DNA markers were separated on 1% TAE agarose gels containing 0.5mg/ml ethidium bromide and photographed on a trans-illuminator. The gel was then treated as for a genomic DNA gel.

Reagents:**Denaturing solution**

1.5 M NaCl

0.5 M NaOH

adjust to 1l with ddH₂O

store at room temperature

Neutralising solution

1 M Tris base

1.5 M NaCl

adjust to pH 7.4 with conc. HCl

adjust to 1l with ddH₂O

store at room temperature

2.2.14 DEPC treatment of solutions used for RNA work.

ddH₂O used for the preparation of solutions for RNA work was treated with 0.1 % (v/v) diethyl pyrocarbonate (DEPC) to inactivate contaminating RNases. DEPC was added and allowed to stand for 30 minutes prior to sterilisation by autoclaving. All plasticware used in the isolation or subsequent work with RNA was also RNase free.

2.2.15 RNA extraction.

TRIzol Reagent (GibcoBRL) was used to isolate total RNA. Worms frozen in liquid nitrogen were transferred to RNase free 1.5ml centrifuge tubes and 1 ml of TRIzol preheated to 68°C was added. The samples were incubated for 8 minutes at 68°C. The samples were removed from the waterbath and allowed to cool at room temperature for 2 minutes. To separate the aqueous and organic phases, 0.2 ml chloroform was added, the tubes were vigorously shaken for 15 seconds, and spun at 12000g for 15 minutes (4°C). The aqueous phase was then transferred to a fresh RNase-free tube and 0.5 ml isopropyl alcohol (2-propanol, BDH) was added to precipitate the RNA. The samples were incubated for 15 minutes at room temperature, centrifuged at 12000g for 10 minutes (4°C) and the supernatant

removed. To remove salts from the sample, 1ml of 70 % ice-cold ethanol was added, the tube was spun at 12000g for 15 minutes (4°C) and the supernatant was removed. The RNA pellet was briefly air-dried, and an appropriate volume of DEPC treated ddH₂O was added to resuspend the RNA. To quantify the RNA, an aliquot was run on an agarose-formaldehyde gel alongside a known quantity of RNA markers. For storage, RNase-free sodium acetate to a final concentration of 0.3 M and double the resultant volume of 100 % ethanol were added and the samples were stored at -70°C.

2.2.16 Preparation of RNA for northern blotting.

Total *B. pahangi* RNA (2-5 µg) was used for the production of northern blots. RNA was diluted to the required concentration with DEPC treated ddH₂O, formamide was added to 50% (v/v) and ethidium bromide to 50 ng/ml. RNase-free 10 X DNA loading dye was added to the samples (final concentration 1X) which were then denatured at 65°C for 10 minutes and loaded onto 1.2 % agarose gels (1 X MOPS) containing 17% formaldehyde. RNA markers (GibcoBRL, 0.24-9.5 kb ladder) were also loaded with the samples and the gel was photographed along side a ruler before blotting to allow size orientation.

Reagents :

10 X MOPS: 0.2 M 3-morpholinopropanesulfonic acid, 50 mM sodium acetate, 10 mM EDTA pH 5.6

2.2.17 Southern and northern blotting.

A gel containing separated DNA was immersed in denaturing solution for 30 minutes, rinsed and immersed in neutralising solution for 30 minutes. The transfer of DNA and RNA to a solid support was by the standard method of Southern (1975) using 20 X SSC [160]. DNA and RNA was immobilised and cross-linked onto nylon membrane by exposure to 150 mJoules of UV radiation.

Reagents:

20 X SSC

3 M NaCl (175.3g)

0.3 M Sodium citrate (88.2g)

800 ml ddH₂O

adjust to pH 7.0 with conc. NaOH

adjust to 1L with ddH₂O

store at room temperature

2.2.18 First strand cDNA synthesis

Unless otherwise stated all reagents used were produced by GibcoBRL. A sample was prepared containing 1-2 µg *B. pahangi* RNA, 1µl of 10 X DNase buffer and 1µl of DNase in a volume of 9 µl. (DEPC ddH₂O was used to adjust the sample volume). The sample was incubated at room temperature for 15 minutes to digest contaminating genomic DNA. Next, 1 µl of 20mM EDTA was added and the sample was incubated at 65°C for 10 minutes, to inhibit enzyme activity. 200 ng of adapted oligo (dT) primer (Table 2.1) and DEPC treated ddH₂O was added to adjust the volume to 12 µl. The sample was then denatured at 70°C for 10 minutes and quick chilled on ice. After a brief centrifugation, 4 µl of 5 X first strand buffer, 2 µl 0.1M DTT and 1 µl 10 mM dNTP mix (10 mM each dNTP at neutral pH) were added. The

contents were mixed gently and incubated at 42°C for 2 minutes. 1 µl (200 units) of Superscript II reverse transcriptase was added and mixed by gently pipetting. The reaction was incubated at 42°C for a further 50 minutes. The reaction was inactivated by incubation at 70 °C for 15 minutes. To remove any RNA complimentary to the cDNA, 1 µl (2 units) of *E. coli* RNase H was added and the solution incubated at 37°C for 20 minutes. The first strand cDNA was then stored at -20°C until required.

2.2.19 Random (High Prime) labelling and purification of DNA probes.

The method was essentially that outlined in the Boehringer Mannheim protocol [161, 162]. 25 ng of purified double stranded DNA was denatured in boiling water for 10 minutes and then kept on ice for 5 minutes. High Prime mix (4 µl), (containing random oligonucleotides, 1 unit/µl Klenow polymerase, 0.125 mM dATP, 0.125 mM dGTP, 0.125 mM dTTP) and 50 µCi of ³²Pα-dCTP (5 µl of 3000mCi/ml) were added to the DNA and the sample was incubated at 37°C for 35 minutes. The reaction was terminated with the addition of 2 µl 0.2 M EDTA. A Nick column (Sephadex G-50, Pharmacia) was used to separate free nucleotides from the radiolabelled probe. The column was equilibrated with TE buffer and the probe solution was added to the pre-wet column. The column was flushed with 400 µl of TE and the radiolabelled probe was eluted by the addition of a further 400 µl of TE. 1 µl of the probe was tested to ensure an activity >6 x 10³cpm/µl. Before addition to the pre-hybridisation solution (2.5.2) the probe was denatured in boiling water for 10 minutes.

2.2.20 Automated sequencing

Double-stranded sequencing reactions were carried out by a method modified from that of Sanger *et al* (1979) [163]. Automated sequencing was performed on a LI-COR model 400 DNA sequencer, which uses fluorescent labelled oligonucleotide primers. Primers were produced by MWG Biotech and corresponded to bacterial vector polylinker sequences.

4 % acrylamide gels were poured as described in the LI-COR sequencing bulletins for the Model 4000 Automated DNA Sequencer. The reagents and protocol were as described in the SequiTherm EXCEL II Long-Read DNA Sequencing Kit-LC product information (Epicentre Technologies). For each sample, four 0.5ml microcentrifuge tubes were prepared, each containing 2 µl of either SequiTherm EXCEL II Termination Mix A, T, C or G. The reaction mix was then equally divided between the four tubes (4 µl to each), the samples were overlaid with mineral oil and placed in a Hybaid touchdown thermocycler for cycle sequencing.

Reaction mix

0.5-1.0 µg DNA (plasmid) template.

2 pmoles of IRD800 labelled primer.

7.2 µl of 3.5 X SequiTherm EXCEL II Sequencing buffer.

1µl of SequiTherm EXCEL II DNA Polymerase.

ddH₂O to 17µl

Cycle sequencing

95°C for 5 minutes		1 cycle
95°C for 30 seconds	}	
50°C for 30 seconds	}	30 cycles
70°C for 1 minute	}	

On completion, 4µl of SequiTherm Stop Solution was added. Immediately prior to loading, the DNA samples were denatured at 95°C for 5 minutes and placed on ice. Typically 0.8 µl of each sample was loaded for sequencing. The sequence data was captured automatically and was downloaded and transferred to the local UNIX system for analysis using the Wisconsin Sequencing Analysis Package (GCG).

Reagents:

TBE buffer (10X)	Sequencing gel solution (6%)
(890 mM Tris-borate, 20mM EDTA)	21 g urea
107.8 g Tris base	6 ml of 10 X TBE buffer
55.0 g boric acid	6 ml of Long Ranger 50 % gel
7.4 g EDTA	concentrate
adjust to 1 L with ddH ₂ O	(FMC Bioproducts, Rockland USA)
	ddH ₂ O to 50 ml
	stir until dissolved

The gel was polymerised with the addition of 25µl of N,N,N',N'-tetramethylenediamine (TEMED) and 250 µl of 10 % ammonium persulphate (APS)

2.2.21 Genotypes of bacterial strains used.

SOLR strain^a *e14⁻(McrA⁻) Δ(mcrCB-hsdSMR-mrr)171 sbcC recB recJ uvrC*

umuC::Tn (Kan^r) lac gyrA96 rel A1 thi-1 endA1 λ^R [F' proAB

lacI^q ZΔM15] su⁻ (nonsuppressing)

XL1-Blue MRF'

Δ(mcrA) 183 Δ(mcrCB-hsdSMR-mrr) 173 endA1 supE44 thi-1

recA1 gyrA96 relA1 lac [F' proAB lacI^q ZΔM15 Tn10 (Tet^r)]

INVαF'

endA1 recA1 hsdR17 (rk⁻, mk⁺) supE44 thi-1 gyrA96 relA1

φ80lacZΔM15 Δ(lacZYA-argF)U169 λ⁻

DH5α

F' φ80dlacZΔM15 Δ(lacZYA-argF)U169 deoR recA1 endA1

hsdR17(rk⁻,mk⁺) phoA supE44 λ⁻ thi-1 gyrA96 relA1

XL1-Blue MRA

D(mcrA) 183 Δ(mcrCB-hsdSMR-mrr) 173 endA1 supE44 thi-1

gyrA96 relA1 lac^c

TOPP

Rif^r [F' proAB lacI^r ZΔM15 Tn10 (Tet^r)]

2.2.22 Analysis of *mmc-1* 5' upstream region.

2.2.22.1 Screening of a *B. pahangi* genomic library.

A *B. pahangi* genomic DNA library in λ-DASH II (Fiona Thompson, University of Glasgow) was screened using a *mmc-1F1-mmc-1R1* gene specific genomic DNA probe. 1 x 10⁶ pfu of the lambda library was plated on NZY plates using XL1-Blue MRA cells (2.2.21) as described for screening a cDNA library (2.5). The plaques were transferred to Hybond-C nitrocellulose membranes and then hybridised at 55°C and washed to 1 x SSC, 0.1 % SDS as described in section 2.5.

2.2.22.2 Cloning of *mmc-1* upstream region by PCR.

Long range PCR was conducted using *AGSGold* DNA polymerase (Hybaid-AGS). PCR was conducted on a *B. pahangi* genomic DNA library in λ -DASH II (Fiona Thompson, University of Glasgow) using nested gene specific primers, *mmc-1gsp1*, *mmc-1gsp2* and *mmc-1gsp3* with vector primers T3 and T7 (see Table 2.1 for primer sequences). It has been shown that high A+T composition of a target DNA sequence may impair the amplification of long PCR fragments under standard conditions [164]. In an attempt to optimise the PCR conditions the extension temperature was lowered from 72°C to 60°C which resulted in amplified bands. The conditions used for the long-range PCR amplification are shown below.

PCR mix for 50 μ l reaction (*AGSGold* polymerase).

	per reaction	final concentration
10 X polymerase buffer	5 μ l	75 mM Tris-HCl,
(<i>AGSGold</i> 750 mM Tris-HCl		pH9, 20 mM (NH ₄) ₂ SO ₄ ,
pH9, 200 mM (NH ₄) ₂ SO ₄ , 15 mM MgCl ₂ ,		1.5 mM MgCl ₂
0.1% Tween 20)		0.01 % Tween 20
dNTPs (10mM each, Perkin Elmer)	2 μ l each	400 μ M each
<i>AGSGold</i> DNA polymerase	0.25 μ l	1.25 units
Primer 1 (50 μ M)	0.5 μ l	0.5 μ M
Primer 2 (50 μ M)	0.5 μ l	0.5 μ M
Template DNA	n μ l	200 ng
ddH ₂ O	to 50 μ l	

Denaturation	94°C	1 minute.	}
Annealing	50°C	1 minute.	} x 30 cycles
Extension	60°C	10 minutes	}

100 µl of the *B. pahangi* λ-DASH II library was boiled for 5 minutes then placed on ice. 5 µl of the boiled library was used in each 50 µl PCR reaction with *mmc-1gsp1* and either T7 or T3 vector specific primers using the conditions described above. After 30 cycles of amplification no amplified products could be detected by EtBr staining. 1 µl of the PCR was then used as template for a nested PCR using *mmc-1gsp2* and the same vector primer as used before. Following this round of nested PCR a number of bands were observed by EtBr staining. To increase the specificity of amplified products a further round of nested PCR was conducted with 1 µl of PCR products using *mmc-1gsp3* and the corresponding vector primer. The specific bands produced after this round of nested PCR were then gel-purified, cloned into pCR 2.1 TA vector (Invitrogen) (2.2.7) and sequenced (2.2.20).

2.3 Production of mammalian-derived mf cDNA library.

2.3.1 Generation of mammalian-derived mf cDNA

Three groups of approximately 1 x 10⁶ mf collected from the peritoneal cavity of jirds were purified from contaminating host cells using standard methods (2.1.4). Following lymphoprep purification the mf were collected by centrifugation (1000g, 5 minutes) and washed twice in 10 ml of mammalian medium (37°C MEM + 10% FCS). The groups of mf were then cultured separately for four hours prior to snap freezing in liquid nitrogen. All manipulations were optimised to avoid prolonged

exposure to ambient conditions. RNA was isolated from each group by standard protocols (2.2.15) and stored at -70°C until use. First strand cDNA synthesis was conducted (2.2.18) from each batch of RNA using 200 ng of adapted oligo (dT) (Table 2.1) per reaction. Two separate hot start PCRs (2.2.2) were conducted utilising each first strand cDNA as a template, using adapter primer, (AP) and SL1 primer (Table 2.1) as shown below.

Denaturation:	94°C	1 minute	}
Annealing:	55°C	1 minute	} x 10 cycles
Extension:	72°C	3 minutes	}
Extension	72°C	10 minutes	

Products from the six resultant PCRs were mixed and then size separated using a Sepharose 400 column, following manufactures protocol (Pharmacia). cDNAs of greater than 400 bp were collected into 1 X PCR buffer (Perkin Elmer). 2 µl of this fraction was then used in four 20 cycle PCRs using the same conditions as shown above. The resulting cDNA was pooled, quantified by an EtBr plate assay (2.6.1) and stored at -20°C until use.

2.3.2 Ligation of cDNA to predigested Uni-ZAP XR vector and packaging of library

10 µg of mammalian-derived mf cDNA (2.3.1) was digested using *EcoR* I and *Xho* I (Pharmacia) in 2 X One-Phor-All buffer (Pharmacia final concentrations,

20 mM Tris-Acetate (pH 7.5) 20 mM magnesium acetate and 100 mM potassium acetate) for 2 hours at 37°C, the reaction was then heat inactivated by incubation at 65°C for 20 minutes. The digested cDNA was again size separated with a Sepharose 400 column (Pharmacia) and quantified by an EtBr plate assay (2.6.1).

150 ng of digested cDNA was ligated into the Uni-ZAP XR vector predigested with *EcoR* I and *Xho* I (Stratagene) at 4°C for 48 hours under the following conditions.

1 µg Uni-ZAP XR vector predigested with *EcoR* I and *Xho* I

150 ng Insert cDNA

1 X Ligase buffer (GibcoBRL)

1 mM ATP (pH 7)

2 Weiss units T4 DNA ligase (GibcoBRL)

ddH₂O to 5 µl

1 µl of the ligation reaction was packaged using Gigapack III gold packaging extract (Stratagene) following the manufactures protocol.

2.3.3 Calculation of titre and percentage recombinants of the primary library

The percentage of recombinant phage in the primary library was calculated by plating the library at 1/10 and 1/100 dilutions on NZY agar plates and conducting blue-white colour selection. 1 µl of the primary library diluted 1/10 and 1/100 in SM Buffer was added to 200 µl of XL1-Blue MRF' cells (2.2.21) grown until OD₆₀₀ = 0.5 and incubated at 37°C for 15 minutes. To this was added 3 ml of molten NZY "top" agar with 15 µl of 0.5 M IPTG and 50 µl of 250 mg/ml X-gal and the cells were plated on NZY agar plates. When the agar had solidified the plates were inverted and

incubated at 37°C overnight. The number of plaques were counted and the titre of the library was calculated in pfu/ml. The percentage of recombinant phage was determined by the percentage of blue (non-recombinant) to white (recombinant) plaques.

The titre of the library was calculated by plating the library as described above in serial dilutions of 1/100 to 1/100000 and counting the number of plaques on non-confluent plates.

Reagents:

SM buffer

- 5.8 g NaCl
- 2.0 g MgSO₄·H₂O
- 50 ml of 1M Tris-HCl pH 7.5
- 5 ml of 2% (w/v) gelatin
- adjust to 1l with ddH₂O
- autoclave and store at room temperature

NZY agar

- 21 g NZY (Difco)
- 15 g BACTO-Agar (Difco)
- 10 mM MgSO₄
- ddH₂O to 1 L
- autoclave, cool and pour plates

NZY "Top" agarose

- 21 g NZY
- 7.5 g Agarose
- 10 mM MgSO₄
- ddH₂O to 1 L
- autoclave, cool to 50°C

X-gal 250mg/ml

Dissolve X-gal in dimethylformamide,
wrap in aluminium foil and store at -20°C

IPTG stock

500 mM isopropyl β-D-thiogalactopyranoside (Sigma) in sterile ddH₂O, store at 4°C.

2.3.4 Amplification of primary library

A high titre stable stock of the library was made following the manufacturers protocol (Stratagene). 1.4×10^6 pfu of the primary library was plated on 25 x 130mm NZY agar plates using MRF' plating cells. The plaques were grown at 37°C for 6 hours then overlaid with 10 ml of SM buffer (2.3.3) per plate and incubated at 4°C for 20 hours with gentle agitation. The SM buffer was recovered and the agar washed with a further 5 ml SM buffer per plate. The collected bacteriophage suspension was pooled in polypropylene centrifuge tubes and chloroform added to a final concentration of 5 % (v/v). Following a 15 minute incubation at room temperature the cell debris was removed by centrifugation at 500g for 10 minutes. The supernatant was collected and chloroform added to a final concentration of 0.3% (v/v). The titre of the amplified library was calculated as described above. 7 % DMSO (v/v) was added to aliquots of the library and these were stored at -70°C.

2.4 Production of mammalian-derived and vector-derived mf cDNAs.

2.4.1 Mf exsheathment and purification.

As part of the vector-derived culture system mf were artificially exsheathed and purified. Mf were washed from the peritoneal cavity of an infected jird and purified (2.1.4). Approximately 1×10^6 mf were resuspended in 2 ml of HBSS and added to a sterile solution of 2 mg/ml Pronase (Protease type XIV from *Streptomyces griseus*, Sigma) solution in HBSS to give a final concentration of 1 mg/ml. The mf were exsheathed for 10 minutes at room temperature with gentle agitation to reduce clumping. The exsheathed mf were collected by centrifugation at 1000g for 5 minutes and washed twice in 10 ml HBSS + 10 % FCS followed by a final wash in HBSS

alone and resuspended in 2 ml Grace's medium (GibcoBRL). The recovered mf were examined by phase contrast light microscopy and approximately 99 % were exsheathed. To isolate the viable mf an agarose pad purification technique was employed [165]. The mf were added to a 35 mm petri dish containing 2 ml of 2 % molten low melting point (LMP) agarose (GibcoBRL) to make a final concentration of 1 % and allowed to solidify. The agarose pad containing mf was overlaid with 1 ml of Grace's medium and incubated at 28°C for 1 hour, after which the Grace's medium containing mf, was removed and replaced with fresh medium (1ml) and incubated for a further hour.

2.4.2 Preparation of cDNA probes.

Mf collected from infected jirds were purified (2.1.4). The mf were then split into two groups and cultured for 16 hours under either mammalian or vector-like conditions. Unless otherwise stated all culture media was obtained from GibcoBRL. For *in vitro* mammalian-derived mf the parasites were cultured at 37°C (5% CO₂) in minimal essential medium (MEM) plus 10% FCS. The *in vitro* vector-derived mf were first exsheathed and purified through agarose pads as described above then cultured at 28°C (5% CO₂) in Grace's insect medium (unsupplemented). To maintain sterility, all manipulations were conducted using standard sterile techniques in a laminar flow hood. The mf were cultured in 2ml of appropriate culture media at a concentration of approximately 5×10^3 mf ml⁻¹, in Nunclon flat-sided tissue culture tubes. Following incubation RNA was isolated and reverse transcribed using an oligo (dT) adapter primer (2.2.18) and stored at -20°C.

2.5 Screening of mammalian-derived mf cDNA library.

2.5.1 Plating the mammalian-derived mf cDNA library.

XL1-Blue MRA (Stratagene 2.2.21) plating cells were grown in L broth (supplemented with 10 mM MgSO₄ and 0.2% maltose) until the OD₅₅₀ equalled 0.8. The cells were centrifuged at 1200 rpm for 15 minutes at 4°C, then resuspended in 10 mM MgSO₄ to achieve a final OD₅₅₀ of 1. 600 µl of the MRA cells were added to 2 x 10³ pfu of the primary library and incubated at 37°C for 15 minutes. 8 ml of "top" agarose (2.3.3) at 50°C was added to the cells and then plated on 130mm NZY agar plates (2.3.3). The "top" agarose was allowed to solidify then the plates were inverted and incubated at 37°C overnight.

2.5.2 Differential screening of the mammalian-derived mf cDNA library

In the primary screen, ten 130mm plates, each with 2 x 10³ pfu of the primary library were plated using MRA on NZY agar plates and grown overnight at 37°C. The NZY agar plates were chilled at 4°C for 2 hours and then overlaid with Hybond-C filters (Amersham). The filters were orientated using needle pricks through the filter and into the agar and then lifted off. The first filter lift was left for 2 minutes and the duplicate lift for 4 minutes. The filters were submerged for 2 minutes in denaturing solution, then for 5 minutes in neutralising solution and finally rinsed briefly in a 2 X SSC solution. The DNA was fixed onto the filters by exposing them to 150 mJoules of UV radiation in a UV cross-linker (Bio-Rad).

DNA probes generated from either mammalian-derived or vector-derived mf cDNA radiolabelled with ³²Pα-dCTP by random primed DNA labelling (2.2.19), were used to hybridise filters at 65°C. The filters were washed to 0.1 X SSC, 0.1%

SDS, and then rinsed with 2 X SSC and exposed to X-ray film. Each positive plaque was cored from the NZY agar and allowed to dissociate into SM buffer by gentle agitation. The secondary screen was conducted at 5×10^2 pfu per 130 mm NZY plate. The differential hybridisation of the positive plaques was tested following PCR amplification of the cloned insert using two vector specific probes and subsequent southern blotting.

Reagents:

SM buffer	20 X SSC
5.8 g NaCl	3 M NaCl (175.3g)
2.0 g MgSO ₄ ·H ₂ O	0.3 M Sodium citrate (88.2g)
50 ml of 1M TRis-HCl pH 7.5	800 ml ddH ₂ O
5 ml of 2% (w/v) gelatin	adjust to pH7.0 with conc. NaOH
adjust to 1 L with ddH ₂ O	adjust to 1 L with ddH ₂ O
autoclave and store at room temperature	store at room temperature
Denaturing solution	Neutralising solution
1.5 M NaCl	1 M Tris base
0.5 M NaOH	1.5 M NaCl
adjust to 1 L with ddH ₂ O	adjust to pH 7.4 with conc. HCl
store at room temperature	adjust to 1 L with ddH ₂ O
	store at room temperature
Hybridisation solution	Wash solutions
6.25 ml 20 x SSC	5-100 ml 20 X SSC
2.5 ml Denhardt's solution	10 ml 10% SDS
1.25 ml 10% SDS	adjust to 1 L with ddH ₂ O
15 ml ddH ₂ O	store at room temperature
heat to hybridisation temperature	pre-heat before use
add 50 µl heat denatured salmon sperm DNA (10mg/ml)	
use immediately	

Denhardt's solution (50 X)

1 % (v/v) Ficoll (type 400)

1 % (v/v) polyvinyl pyrrolidone

1 % (w/v) bovine serum albumin fraction V

adjust to 100 ml with ddH₂O

aliquot and store at -20°C

2.5.3 *In vivo* excision of the pBluescript phagemid from the Uni-Zap vector.

The Uni-Zap XR vector used for the construction of the cDNA library (2.3) allows the excision of the cloned insert to form a circular phagemid that can be used for further manipulations. The protocol given is that from the Uni-Zap XR vector kit manual (Stratagene). Phage in SM buffer (2.5.2), were used to inoculate 200 µl of a culture of XL1-Blue MRF' cells (Stratagene 2.2.21) at an OD₆₀₀. To this >1 x 10⁶ pfu ExAssist helper phage were added and grown in L broth (2.2.7) supplemented with 0.2 % (w/v) maltose and 10mM MgSO₄ for 3 hours at 37°C. The culture was heated at 70°C for 20 minutes and then centrifuged at 1000g for 15 minutes. The supernatant contained the excised phagemid. The phagemid could then be plated on L-amp plates (2.2.7) using freshly grown SOLR plating cells (Stratagene 2.2.21).

2.6 Analysis of gene expression by semi-quantitative RT-PCR.

Semi-quantitative RT-PCR determines the level of expression of a gene of interest compared to that of a constitutively expressed endogenous control gene [166]. For these studies the gene encoding *β-tubulin* was used as the constitutive control gene.

2.6.1 Ethidium Bromide plate assay for quantitation of cDNA.

To determine the concentration of samples of first strand cDNA prior to analysis of expression by RT-PCR samples were compared to a series of standards by UV visualisation. The protocol used is taken from Appendix VI from the UNI-Zap library construction protocol (Stratagene).

100 ml of 0.8 % (w/v) molten agarose was prepared using TAE buffer and cooled to 50°C before the addition of 10 µl EtBr (10 mg/ml). Approximately 10 ml of the mixed agarose was then poured into 90 mm petri dishes and allowed to dry. Dilutions of a known DNA standard were made in the range 100 ng/µl to 2 ng/µl. The underside of the petri dish was labelled with the positions where the unknown cDNA and the standards were to be spotted. 0.5 µl of each of the standards and the sample cDNA were carefully spotted onto the surface of the agarose. After the samples had been absorbed into the agarose (10-15 minutes room temperature) the plate was inverted and photographed over UV light. The concentration of the unknown sample can be directly compared to that of the standards. The plates can be stored in the dark at 4°C for 1 month, but should not be reused.

2.6.2 Preparation of cDNA panel from different life-cycle stages for RT-PCR analysis.

Different life-cycle stages of *B. pahangi* from the mammalian host were isolated from infected jirds on the appropriate days post-infection and purified as described in 2.1.4. Total RNA isolation (2.2.15) and first strand synthesis (2.2.18) was conducted as described earlier.

To extract RNA from parasite stages in the mosquito (L1-infective L3) fed females at the appropriate day post-feed were picked and the thorax dissected and stored in liquid nitrogen. The RNA extraction was conducted essentially as described for standard RNA extraction (2.2.15) with the following modifications.

The frozen thoraces were lysed at 68°C for 10 minutes in 250 µl lysis buffer containing proteinase K (500 µg µl⁻¹ final concentration), 25 µl β-mercaptoethanol and homogenised using a disposable micropestle (Eppendorf), prior to the addition of the pre-warmed TRIzol reagent. This was found to increase the quantity of RNA extracted [77].

Reagents:

Lysis buffer (10 ml)

(0.1 M Tris-HCl, 0.2 M NaCl, 2 % SDS, 0.2 M EDTA)

1 ml of 1 M Tris-HCl pH 8.0

1 ml of 2 M NaCl

2 ml of 10 % SDS

4 ml of 0.5 M EDTA pH 8.0

2 ml of DEPC ddH₂O

prepared fresh for each RNA extraction

2.6.3 Titration of RT-PCR amplified products.

In order to compare the differences in amplification of the gene of interest and control gene products, the PCR reaction must be stopped while it is still in the exponential stage of amplification. To determine the optimal number of cycles for each of the primers sets 100 µl PCR reactions were prepared in the standard manner for each of the genes of interest and the constitutively expressed control gene β-*tubulin*. 10, 15, 20, 25, 30, and 35 cycles of PCR were conducted, 10µl of the

reaction was removed, soaked at 72°C for 10 minutes to allow complete amplification, and these products were then separated on 2% agarose gels and blotted onto nylon membrane and probed with the appropriate ³²P labelled cDNA. The degree of hybridisation was calculated by overlaying the developed autoradiograph with the corresponding Southern blot and excising a 0.5 cm square of the filter corresponding to the specific band. Each piece of nylon membrane was placed in 3 ml of scintillation fluid (Optiscint) and the β-emissions counted. The results were graphed to determine the optimal number of PCR cycles.

2.6.4 Semi-quantitative RT-PCR analysis.

RNA was isolated from different parasite life-cycle stages or from infected mosquito thoraces and reverse transcribed with oligo (dT) by standard methods (2.2.18). The first strand cDNA was then PCR amplified with gene specific primers. 10 µl of the resulting PCR products were separated on 2 % agarose gels and transferred to nylon membrane by standard methods. The blots were then probed at high stringency with the corresponding gene-specific cDNA and exposed to autoradiographic film. The degree of hybridisation was calculated as described above (2.6.2). Relative expression at each stage was determined by calculating the ratio of the gene of interest (minus background counts) compared to the constitutively expressed β-*tubulin* gene (minus background counts).

2.7 5' RACE

To obtain additional sequence information 5' to the cloned fragment of *mmc-1* and to confirm the presence of the SL1 spliced leader sequence 5' RACE was carried out. The procedure was conducted following the manufacturers protocol (5' RACE system for Rapid Amplification of cDNA Ends, Version 2.0, GibcoBRL). Total RNA was isolated by standard methods (2.2.15), from approximately 1 x 10⁶ mf of *B. pahangi* from the peritoneal cavity of an infected jird. First strand cDNA was synthesised by standard methods (2.2.18) using *mmc-1*gsp1 primer (Table 2.1). The first strand cDNA was purified by the GlassMAX DNA isolation spin cartridge system (GibcoBRL), and then tailed with dCTP by terminal deoxynucleotidyl transferase (TdT) as outlined below.

TdT Tailing reaction mix

DEPC treated ddH ₂ O	6.5 µl
5 X tailing buffer	5.0 µl
2 mM dCTP	2.5 µl
GlassMAX purified cDNA	10 µl
Final composition of reaction, 10 mM Tris-HCl (pH 8.4), 25 mM KCl, 1.5 mM MgCl ₂ , 200 µM dCTP	

The TdT tailing reaction mix was incubated for 2 minutes at 94°C, then chilled for 1 minute on ice. To this 1 µl of TdT was added and then incubated at 37°C for 10 minutes. TdT was inactivated by incubation at 65°C for 10 minutes.

The first round of nested amplification used the abridged anchor primer (AAP) and *mmc-1gsp2*. The PCR was conducted with Taq DNA polymerase as described previously (2.2.1). The protocol for the amplification was as shown below.

Denaturation	94°C	2 minutes	}
Annealing	55°C	1 minute	} x 35 cycles
Extension	72°C	2 minutes	}
Final extension	72°C	10 minutes	

The resulting cDNA was diluted 1/100 and a further nested PCR was conducted using the abridged universal anchor primer (AUAP) and *mmc-1gsp3* exactly as described above. The resulting amplified 5' fragment was ligated into pCR2.1 TA vector (Invitrogen) then transformed into Oneshot INVαF' cells (2.2.7) and sequenced (2.2.20).

2.8 Culture of *B. pahangi* parasites *in vitro*.

Mf collected from the peritoneal cavity of infected jirds represent a heterogeneous population of various ages. In order to determine the expression of *mmc-1* of mf released within a defined time span, *B. pahangi* adults were cultured *in vitro* and the released mf were collected at set time points. Adults were cultured in RPMI + supplements (RPMI+) or in no glucose RPMI medium (RPMI-g) at 37°C in 5 % CO₂. Modifications to the medium in individual experiments are given in the text. Unless otherwise stated the cultures were conducted in 25 cm² culture flasks (CoStar). Following culture the adults were removed with a sterile glass hook. The mf were collected by centrifugation at 1000g for 5 minutes then frozen in liquid nitrogen until required.

RPMI +	RPMI-g
RPMI 1640 (Dutch modification)	RPMI 1640 (Without L-glucose)
10 % FCS	10 % FCS
1 % Glucose	2 mM L-glutamine
2 mM L-glutamine	2.5 mM Hepes
2.5 mM Hepes	100 U/ml penicillin
100 U/ml penicillin	100 µg/ml streptomycin
100 µg/ml streptomycin	

2.9 Production and purification of MBP-MMC-1 fusion protein.

The pMal protein fusion and purification system (New England Biolabs) was used to produce the fusion protein as described in the manufacturers protocol. By cloning the *mmc-1* ORF downstream of the *malE* gene which encodes the maltose binding protein (MBP), expression leads to the production of an MBP-MMC-1 fusion protein. The possession of the MBP moiety allows the purification of the fusion protein by affinity chromatography on an amylose column, following which the protein is then eluted by the addition of a buffer containing maltose.

2.9.1 Cloning of MMC-1 downstream of the *MalE* gene.

Two PCR primers were designed (MMC-1ExF1 and MMC-1ExR1). MMC-1ExF1 begins at the ATG initiation codon and contained 7 nucleotides of the ORF while MMC-1ExR1 incorporated the last 7 nucleotides of the ORF and the stop codon. Each primer possessed a GCCG clamp to increase the specificity of annealing in further rounds of PCR. A specific restriction site was also included that allowed subsequent directional cloning of the PCR fragment into the MBP vector. PCR amplification of mammalian-derived mf first strand cDNA was conducted at low

stringency (50°C, annealing temperature). Following 30 cycles of PCR no amplification could be observed, so a 1 µl aliquot was removed and re-amplified as before. A band of predicted size (266 bp) was observed which was gel-purified and ligated into a TA vector (pCR2.1 Invitrogen TA::MMC-1ExF1R1).

2.9.2 Subcloning of MMC-1 into pMal-p2.

The pMal-p2 plasmid provided in the kit was transformed into competent *E. coli* cells (DH5α GibcoBRL) and grown overnight at 37°C in LB-broth supplemented with ampicillin (LB-amp 100 µg/ml). The TA::mmc1ExF1-R1 clone was also grown overnight in LB-amp. Both plasmid DNAs were isolated by standard procedures and digested for three hours with both *Bam* HI and *Pst* I restriction enzymes. The resulting gel purified DNAs were ethanol precipitated and ligated together. The ligated plasmid (MBP-MMC-1) was then transformed into DH5α cells and plated on LB-amp agar plates containing X-gal.

2.9.3 Transformation of Topp competent cells.

To allow the expression of high titre fusion protein the MBP-MMC-1 plasmid was transformed into highly competent Topp cells (Stratagene). To render the Topp cells competent a 10 ml aliquot of L broth (with no antibiotics) was inoculated with 10 µl of Topp cell glycerol stock and grown overnight at 37°C. 1 ml of this culture was added to 40 ml of fresh L broth and grown for a further 2.5 hours. The cells were collected by centrifugation at 3000 rpm for 10 minutes at 4°C, then resuspended in 20 ml of ice cold 50 mM CaCl₂ and incubated on ice for 30 minutes. The cells were then re-centrifuged (3000 rpm for 10 minutes at 4°C) and resuspended in 2 ml ice

cold 50 mM CaCl₂. The cells were then either used immediately or were stored at 4°C overnight.

1 µl of a 10-fold dilution of the MBP-MMC-1 plasmid miniprep (2.2.9) was added to 200 µl of competent Topp cells and incubated for 30 minutes on ice, 2 minutes at 42°C and 10 minutes at room temperature. 1 ml of L broth was added and the cells were incubated at 37°C for 1 hour (225 rpm horizontal shaker). The cells were collected by centrifugation at 2000 rpm for 3 minutes and the media removed to approximately 200 µl. The cells were gently resuspended without pipetting and then plated on LB agar plates containing ampicillin (100 µg/ml).

2.9.4 Large scale expression of MBP-MMC-1

Two 10 ml aliquots of LB broth containing 100 µg/ml final concentration of ampicillin (LB-amp) were inoculated with 50 µl each of a MBP-MMC-1 plasmid glycerol stock (2.2.10) and grown at 37°C in a horizontal shaker at 225rpm overnight. Both were then added to 1 litre of LB-amp and grown for a further 3 hours as before. A 1 ml aliquot was removed at this stage and labelled "uninduced". 3 ml of freshly made 100 mM IPTG was then added and the culture grown for 4 hours at 37°C as before, following which a further 1 ml aliquot was removed and labelled "induced". The remaining induced cells were centrifuged in a Beckman J2-21 centrifuge at 8000 rpm for 15 minutes at 4°C, the supernatant removed and the cells resuspended in a total of 50 ml of column buffer. The cell suspension was sonicated on ice, at 10 cycles of 20 seconds on, 30 seconds off (Soniprep 150, MSE) until the suspension cleared. The lysed cells were centrifuged at 10000 rpm for 15 minutes the supernatant was carefully removed and frozen at -20°C.

Reagents:

IPTG stock

100 mM isopropyl β -D-thiogalactopyranoside (Sigma) in sterile ddH₂O, store at 4°C.

2.9.5 Affinity purification of the MBP -MMC-1 fusion protein.

An amylose column was prepared in the barrel of a 50 ml syringe stoppered with glass wool to a depth of approximately 1 cm. 20 ml of amylose resin (New England Biolabs) was layered onto the glass wool and washed with eight times the column volume with column buffer (approximately 160 ml). The lysate was diluted 1:1 with column buffer and run through the column. The binding proteins were washed with column buffer until the OD₂₈₀ read zero. The MBP-MMC-1 protein was then eluted from the column by the addition of 30 ml column buffer + 20 mM maltose. The eluted protein was collected in 3 ml fractions and the concentration calculated by spectrophotometry (280 nm).

Reagents:

Column Buffer

20 mM Tris-Cl pH 7.4
200 mM NaCl
1 mM EDTA
Make to 1 L with ddH₂O
Store at 4°C

Column Buffer + 20 mM maltose

20 mM Tris-Cl pH 7.4
200 mM NaCl
1 mM EDTA
20 mM maltose
Make to 1 L with ddH₂O
Store at 4°C

2.9.6 Digestion of MBP-MMC-1 fusion protein and purification of recombinant MMC-1.

The fusion protein eluted from the amylose column (2.9.5) was digested by Factor Xa (New England Biolabs) that specifically cleaves a recognition site in the MBP vector therefore releasing the MMC-1 recombinant protein.

The reaction was conducted at 23°C for 6 hours with occasional mixing.

1 mg MBP-MMC-1

10 µg Factor Xa

1 X Factor X buffer (20 mM Tris-HCl pH 8.0, 100 mM NaCl, 2 mM CaCl₂)

to 500 µl in ddH₂O

To further purify the digested MMC-1 recombinant protein the MMC-1 protein was separated by SDS-PAGE on a 15 % gel (2.11.1) and the MMC-1 band excised. The protein was then eluted from the acrylamide using a BioRad model 422 electro-eluter as described in the manufacturer's protocol. The concentrated MMC-1 protein was dialysed overnight at 4°C against ddH₂O using 3.5 kDa cut off membrane tubing (Spectrum Medical Industries Inc). The purified MMC-1 protein was quantified by the Bradford method (2.15.2), supplemented with 0.02 % sodium azide and stored at -20°C.

2.10 Raising anti-MMC-1 immune sera.

Cut MMC-1 recombinant protein was purified from the MBP moiety by SDS-PAGE. The specific band was excised and washed 3 times in 20 ml ddH₂O then frozen at -20°C. The frozen antigen was pulverised in liquid nitrogen with a pestle

and mortar and then used to raise anti-sera in both BALB/c mice and rabbits. Ten full size SDS gels were used to produce the antigen for the immunisations, where used the adjuvant was Freund's incomplete (Sigma). The rabbit anti-sera was produced by the Scottish Antibody Production Unit (SAPU, Law Hospital Lanarkshire). Two rabbits were immunised on four occasions (day 0, 28, 56 and 84). Terminal exsanguinations were performed on day 91.

A group of 10 BALB/c mice were immunised on four separate occasions (day 0, 21, 39) with 1.5 ml pulverised MMC-1 antigen mixed with 500 µl ddH₂O and 2 ml Freund's incomplete adjuvant. 200 µl of antigen was administered per mouse by subcutaneous injection. The final boost (day 56) was administered into the peritoneum and did not contain any adjuvant. The mice were killed by CO₂ inhalation and bled by cardiac puncture on day 60 into 1ml syringes. The blood was left to clot overnight at 4°C following which serum was collected into Eppendorf tubes and centrifuged at 10,000 rpm for 10 minutes at 4°C. The serum was pooled and then aliquoted and stored at -20°C.

2.11 Immunochemical techniques.

2.11.1 SDS polyacrylamide gel electrophoresis (PAGE)

The Hoefer vertical electrophoresis system was used with either longer-length mini gels (10.1 x 10.6cm) or slab gels (17.9 x 16cm) according to the quantity of protein analysed and the degree of separation required. Electrophoresis was carried out at 20 mA per gel for mini gels, or at 12 mA overnight for larger gels.

For large gels the separating gel solution was mixed and degased for 15 minutes prior to the addition of 300 µl SDS, 100 µl of freshly prepared 10%

ammonium persulphate (APS) and 20 µl of N,N,N',N'-tetramethylethylenediamine (TEMED). The gel solution was pipetted to within 3 cm of the top of the gel apparatus, to allow room for the stacking gel and then carefully overlaid with water-saturated butanol. The gel was left to polymerise for 30-60 minutes. The butanol was rinsed from the gel with ddH₂O and then the gel surface was dried using strips of 3MM filter paper. The stacking gel solution was mixed and degased prior to the addition of 100 µl SDS, 50 µl APS and 10µl of TEMED. The gel was then poured onto the separating gel and an appropriate comb was inserted (as the gel sets more solution may need to be added). When the stacking gel had set, the wells were rinsed with ddH₂O and then with 1 X running buffer. Protein samples were mixed with an equal volume of SDS sample cocktail, boiled for 3 minutes and loaded into the wells of the gel. The gel was run until the dye front had reached the bottom of the apparatus. The gel was removed and the proteins visualised by staining for 20 minutes in Coomassie Blue, the excess stain was removed by immersion in destain solution. The stained gel was dried by immersion for 30 minutes in drying reagent then placed between acetate sheets and dried in an "Easy-Breeze" gel dryer (Hoeffer).

Reagents:

1.5M Tris-HCl pH 8.8

1.5 M Tris base
ddH₂O to 50ml
stir until dissolved
adjust to pH 8.8 with conc. HCl
adjust to 100ml with ddH₂O
store at 4°C

0.5M Tris-HCl pH 6.8

0.5 M Tris base
ddH₂O to 30ml
stir until dissolved
adjust to pH 6.8 with conc. HCl
adjust to 50ml with ddH₂O
store at 4°C

Running buffer pH 8.3 (10X)

0.25 M Tris base

1.92 M glycine

0.1 % SDS

ddH₂O to 1 L

SDS Sample cocktail

Solution (a)

2.5 ml 0.5M Tris-HCl pH 6.8

4 ml 10% SDS

2 ml glycerol

ddH₂O to 10 ml

Solution (b)

0.01g bromophenol blue (BPB)

2.3g dithiothreitol (DTT)

ddH₂O to 10ml

aliquot and store at -20°C

for use: mix 650µl of (a) and 100µl of (b)

(0.108M Tris, 3.5%SDS, 17.3% glycerol, 0.013% BPB, 0.2M DTT)

Coomassie Blue stain

1 g Coomassie brilliant blue

450 ml methanol

450 ml ddH₂O

100 ml glacial acetic acid

filter through Whatman No.1

Gel destain (5 L)

350 ml glacial acetic acid

1 L methanol

3650 ml of ddH₂O

drying reagent

100 ml glacial acetic acid

10 ml glycerol

ddH₂O to 1 L

12.5 % separating gel.

12.5 ml 30% acrylamide solution (protogel)

11.2 ml 1.5M Tris-HCl pH 8.8

6.2 ml ddH₂O

15 % separating gel.

15 ml 30% acrylamide solution

11.2 ml 1.5M Tris-HCl pH 8.8

0.7 ml ddH₂O

3 ml glycerol

5% stacking gel.

1.67ml 30% acrylamide solution

1.25 ml 0.5M Tris-HCl pH 6.8

7.0 ml ddH₂O

(30% acrylamide solution contains 2.7% N,N'-methylene-bis-acrylamide)

2.11.2 SDS-sample cocktail extracts.

Protein extracts of life-cycle stages for western blot analysis were produced from 1×10^5 mf, 100 mixed sex adults or 1×10^4 L3. The parasites were removed from liquid nitrogen, 100 μ l SDS-sample cocktail (2.8.1) was added and then boiled for 3 minutes. The extract was centrifuged for 5 minutes at 13,000 rpm then the supernatant removed.

2.11.3 Molecular weight markers.**Low molecular weight markers (Bio-Rad) 14.4-97.4kD**

lysozyme (14.4kD), trypsin inhibitor (21.5kD), carbonic anhydrase (31.0kD), ovalbumin (45.0kD), serum albumin (66.2kD), phosphorylase B (97.4kD).

Protein standards were diluted 1:20 in SDS sample cocktail prior to use.

Ultra low molecular weight markers (Sigma) 1.06-26.6 kDa

Bradykinin (1.060 kDa), Bovine Insulin chain B, oxidised (3.496 kDa), Apoprotein from bovine lung (6.5 kDa), α -Lactalbumin from bovine milk (14.2 kDa), Myoglobin from horse heart (17 kDa), Triphosphate Isomerase (26.6 kDa).

Protein standards were diluted 1:20 in SDS sample cocktail prior to use.

2.11.4 Staining SDS-Polyacrylamide gels with Silver nitrate.

The technique used is a modification of that described by Sambrook *et al* [159]. The protein samples were separated by standard SDS-PAGE procedures then the proteins fixed by sequential submersion in 5 gel volumes of: 50 % methanol, 10 % Acetic acid; 5 % methanol, 7 % acetic acid; distilled water; 5 µg DTT/ml ddH₂O, each for 20 minutes at room temperature with gentle agitation. The proteins were then stained with 0.1 % silver nitrate in ddH₂O for 20 minutes at room temperature. To develop the gel it was placed in developer solution until sufficient contrast was observed (5-10 minutes). To stop the reaction the gel was immersed in 1 % acetic acid then rinsed in ddH₂O.

Reagents:

Developer solution

3 g Na₂CO₃ (3 %)

50 µl 37 % formaldehyde (0.02%)

make to 100 ml with ddH₂O

2.11.5 Western blotting.

The western blot procedure was a modification of that described by Towbin *et al* [167]. Protein samples and markers (Bio-Rad, Sigma) were run on a SDS-polyacrylamide gel. The proteins were then transferred onto nitrocellulose membrane (BioTrace NT, Gelman Sciences) using the Hoefer mini-blotting system at a constant current of 200mA for 1 hour. The blot was stained for 5 minutes with Ponceau S, the positions of the markers were labelled and the protein lanes numbered with a soft pencil then cut into strips. To block non-specific binding, the sample strips were soaked in TBS/Tween 20 + 5% BSA for 1 hour. The strips were then rinsed three

times for 5 minutes in TBS/Tween 20 and were incubated for 1 hour at room temperature with test serum or control serum at concentrations ranging from 1/100 to 1/800 in PBS + 0.1 % BSA. The strips were rinsed with TBS/Tween 20 and then incubated for 1 hour at room temperature with the secondary antibody, goat anti-rabbit or anti-mouse alkaline phosphatase conjugate (Sigma) diluted 1/6000 in PBS + 0.1 % BSA. The strips were rinsed again and the blot was developed by incubating the strips in BCIP/NPT substrate (Sigma) until adequate staining occurred. To stop the reaction the strips were rinsed with ddH₂O. The blot was dried by blotting between Whatman filter paper and then reformed by re-aligning the strips using the pencil markings.

Reagents:

Transfer buffer

6.05 g Tris base (0.025 M)
28.2 g glycine (0.19 M)
400 ml methanol
ddH₂O to 2 L

Tris buffered saline (TBS)

12.1 g of Tris base (0.05 M)
43.75 g of NaCl (0.15 M)
adjust to pH 7.4 with conc. HCl
ddH₂O to 5 L

TBS/Tween 20

0.05 % Tween 20 (polyoxyethylenesorbitan monolaurate, Sigma) in TBS.

TBS/Tween 20 5 % BSA

Make 100 ml TBS/Tween 20 as above, add 5 g Bovine serum Albumin (Boehringer).

TBS/Tween 20 1 % BSA

Make 100 ml TBS/Tween 20 as above, add 1 g Bovine serum Albumin (Boehringer).

Ponceau S Stain

Dissolve 0.2 g Ponceau S (Sigma) in 100 ml of 3 % trichloroacetic acid solution.

2.11.6 Metabolic labelling of mf and immunoprecipitation of MMC-1.

The protocol for metabolically labelling is essentially that of Devaney *et al* [41]. Mf were recovered from the host by peritoneal lavage and purified from contaminating cells as described in 2.1.4. For labelling, mf were washed with HBSS before being transferred to sterile 1.5ml screwtop microcentrifuge tubes and resuspended in 0.5ml of culture medium. The parasites were incubated at 37°C for 10 minutes prior to the addition of 50 µCi of ³⁵S-methionine (15mCi/ml >1000Ci/mmol, Amersham). Metabolic labelling was carried out for 4 hours and then the radiolabelled methionine was rinsed from the worms. The mf were collected in the bottom of the tube by centrifugation at 1000g for 5 minutes and then resuspended in fresh culture medium. This procedure was repeated twice. Following homogenisation with a micropestle (Eppendorf), protein was extracted from the samples using 200 µl IEF lysis buffer for two hours at room temperature. The samples were centrifuged at 13000g, to pellet insoluble components then the supernatant was transferred to a fresh tube and stored at -20°C. To determine the amount of ³⁵S-methionine incorporation in a parasite extract, a trichloroacetic acid precipitation (TCA) was utilised. Duplicate 2.5 µl aliquots of the ³⁵S-labelled extract were mixed with 10 µl of rabbit serum, 1 ml of ice cold 10 % TCA (Sigma) was added and the solution was incubated on ice for 10 minutes. A conical vacuum flask was used to filter the TCA-precipitated proteins onto Whatman glass fibre filter paper disks. The TCA solution was filtered through the disk and the vial was rinsed out with 1ml of TCA. The precipitant was washed with 1ml of ethanol followed by 1ml of acetone. The protein-coated disks were then transferred to scintillation vials, 3ml of OptiScint scintillation fluid (Wallac) was added and the ³⁵S activity was measured for 60 seconds (cpm) in a

Beckman scintillation counter. 1×10^6 cpm of precipitable protein was separated by SDS-PAGE on a 15 % slab gel and stained with Coomassie Blue. The gel was then placed in drying reagent (2.8.1) for 30 minutes, then in fluorographic reagent (Amplify, Amersham) for 30 minutes before drying under vacuum. The dried gel was then exposed to pre-flashed high-sensitivity X-ray film (Kodak) and stored at -70°C for 4-8 weeks.

Reagents :

Culture medium

Methione free MEM (GibcoBRL)
containing:
1 % glucose (Sigma)
25 mM HEPES
4 mM glutamine
1 % non-essential amino acids
100 mg/ml gentamycin (Sigma)
make up in MEM (minus methionine)
Filter (CoStar) into sterile container
5 % foetal calf serum
store at 4°C

IEF lysis buffer

9.5 M Urea
2 % NP-40
2 % Ampholines pH 3.5-10(Pharmacia)
50 mM DTT
adjust to 10ml with ddH₂O
store as 0.5ml aliquots at -70°C

2.12 Culture of mf for collection of E/S products.

Approximately 2×10^6 mf isolated from the peritoneal cavity of an infected jird were cultured aseptically for 24 hours in 10 ml of RPMI+ medium (2.8). Mf were removed by centrifugation at 1000g for 5 minutes and the medium concentrated 40 fold to approximately 500 μl , using a 5 kDa molecular weight cut-off concentration column (Vivaspin). The E/S products were resuspended in SDS sample cocktail and analysed by SDS-PAGE.

2.13 Effect of MMC-1 IgG on parasite development in mosquitoes.

2.13.1 Isolation of IgG from immunised rabbit serum.

Immunoglobulin G (IgG) was purified from MMC-1 rabbit antiserum (R718, 2.8) or from serum from rabbits immunised with an unrelated protein, cytidine deaminase (CDD) by standard ammonium sulfate precipitation [168]. Briefly the serum was stirred as 0.5 times the volume of saturated ammonium sulphate (pH 7) was added. The serum was incubated at 4°C overnight then centrifuged at 3000g for 30 minutes. The supernatant was removed and discarded and the pellet resuspended in PBS. The IgG was then dialysed against PBS at 4°C overnight in visking tubing (BDH approximate pore size 12.0 kDa). The resulting IgG was quantified (2.15.2) and stored at -20°C.

2.13.2 Feeding mosquitoes.

Aedes aegypti, strain (Ref^m) mosquitoes were fed with rabbit blood containing approximately 350 mf/20 µl supplemented with either 100 µg/ml CDD IgG or 100 µg/ml MMC-1 IgG for one hour using a membrane feeder (2.1.2). The blood containing IgG and mf was then replaced with fresh blood and the mosquitoes fed for a further hour. Fed females were isolated in a netted cage and maintained under standard conditions (2.1.1) for 9 days.

2.13.3 Assessing the development of parasites.

At day 9 post-infection mosquitoes were collected, the wings removed and the body dissected into head, thorax and abdomen on a microscope slide. The three

body sections were then carefully teased apart in a drop of HBSS and the numbers and developmental stage of the parasites were recorded.

2.14 Fluorescent localisation of MMC-1.

The methods used for fluorescent localisation are based on techniques developed for studying *C. elegans* as described by Miller and Shakes (1995) [169] and adapted as described below. All manipulations were conducted using low retention Eppendorf tubes and pipette tips (Axygen Inc).

2.14.1 Staining whole parasites.

To localise the MMC-1 protein in whole intact mf or exsheathed mf (exsheathed by exposure to Pronase, 2.4.1), approximately 1×10^5 mf were added to 15 ml fix solution for 12 hours at 4°C, then the mf were washed three times at room temperature in PBS (5 minutes on rotator, 1000g centrifugation for 5 minutes to collect worms) and stored in storage buffer overnight. The worms were washed in PBS as before then placed in 200 µl of primary antibody diluted in antibody dilution buffer (1/50 or 1/100) and incubated overnight at 4°C. Once again the mf were washed in PBS as before and then transferred to a clean Eppendorf tube. 200 µl of secondary antibody (Fluorescein isothiocyanate (FITC) conjugated goat anti-rabbit IgG, Sigma) at a 1/200 dilution in antibody dilution buffer was added and incubated at 4°C for 4 hours. The mf were washed in wash solution (3 x 15 minute washes), collected by centrifugation (1000g x 5 minutes) and the wash solution removed. The mf were resuspended in 100 µl Evans Blue counterstain. The mf were collected again and excess Evans Blue removed, the mf were then mounted on microscope slides in

Dabco mounting buffer. The mounted worms were examined under an Olympus BX60 UV fluorescence microscope using a BP460-490 excitation filter. Images were recorded using a SPOT photocapture system (Diagnostic Instruments Inc.) Control reactions; no primary antibody, no fluorochrome or with pre-immune serum were conducted to determine background fluorescence.

Reagents:

Fix solution	Storage buffer
4% (v/v) Paraformaldehyde (PFA) in PBS (2.15.6)	0.1% PFA in PBS
Antibody dilution buffer	Wash solution 1
0.1% (w/v) BSA 0.5% (v/v) Triton X-100 in PBS	0.1% (v/v) Triton X-100 in PBS
Evans Blue counterstain	Dabco mounting buffer
0.1% Evans Blue in PBS	Dabco/Glycerol 50 % Glycerol in ddH ₂ O 2.5 % Dabco pH 8.0 (1,4-diazobicyclo-[2.2.2]-octane)

2.14.2 Staining of permeabilised parasites.

To localise MMC-1 antibodies to internal epitopes, exsheathed mf were permeabilised prior to staining. Mf were exsheathed (2.4.1) then fixed and stored as described above. The mf were then incubated overnight at 37°C in 10 ml of BME solution then collected and washed 3 x 5 minutes in PBS (collected by centrifugation 1000g x 5 minutes). The mf were transferred to a fresh Falcon tube containing 10 ml of collagenase buffer and incubated for 4 hours at 37°C in a rotary shaker at 110 rpm.

The mf were washed as before and fluorescent localisation was then conducted as described for whole parasites (2.14.1).

Reagents:

BME solution	Collagenase buffer
5% (v/v) β -mercaptoethanol	1 mM CaCl ₂
1% (v/v) Triton X-100	100 mM Tris-HCl pH 7.5
125 mM Tris-HCl pH 6.9	115 digestion units/ml collagenase (Sigma)

2.15 Analysis of immune response to MMC-1 antigen.

2.15.1 Preparation of soluble extracts.

Adult *B. pahangi* soluble extract for use in the cell culture experiments was prepared by homogenisation on ice in RPMI+ (1640 Dutch modification). The homogenate was incubated on ice for 60 minutes with occasional mixing and then centrifuged at 10,000g for 30 minutes at 4°C. The supernatant was sterilised by filtration through a 0.45 μ m Spin-X filter (CoStar), and assayed for protein concentration by the Bradford method (2.15.2) and stored at -70°C until use.

Soluble extracts of mf were produced from approximately 5×10^6 mf in 500 μ l RPMI+ (1640 Dutch modification). The mf were sonicated on ice (9 x 10 seconds on 1 minute off, Soniprep 150, MSE). The extract was then incubated on ice for a further 30 minutes with occasional mixing. The homogenate was then centrifuged at 10,000g for 30 minutes at 4°C. The supernatant was sterilised by filtration through a 0.45 μ m Spin-X filter (CoStar), and assayed for protein concentration by the Bradford method and stored at -70°C until use.

Reagents:**RPMI+**

RPMI medium (1640 Dutch modification)

5 mM L-glutamine

5 mM Hepes

100 U/ml penicillin

100 µg/ml streptomycin

2.15.2 Protein assay the Bradford method.

The protein concentration of the samples was determined using a BioRad dye reagent based on the Bradford method [170]. A protein sample of unknown concentration was diluted with ddH₂O (typically 1/15), 10-50 µl of sample was added in duplicate to the wells of a flat-bottomed 96-well plate and made to 160 µl with ddH₂O. Standards were prepared using dilutions of BSA (0.5 µg to 5µg) and made to 160µl with ddH₂O. 40 µl of Bradford reagent (BioRad) was added to each well, the samples were mixed and read on a micotiter plate reader at 595nm wavelength after eight minutes. The protein concentrations of the samples were determined from a standard curve produced from the absorbance of the protein standard concentrations.

2.15.3 Animals and infection protocols.

6 week-old male BALB/c mice purchased from Harlan-Olac (Bicester, U.K.) were maintained in filter top cages. Groups of 5 mice were injected twice (day 0, day 14) s.c. with either soluble mf extract (100 µg/mouse in Freund's incomplete adjuvant), MMC-1 antigen (200µl pulverised antigen (2.10) in Freund's incomplete adjuvant) or adjuvant alone. Animals were killed by CO₂ inhalation and the spleens removed.

2.15.4 Preparation of spleen cells.

Spleens were washed in RPMI and teased to a single cell suspension by pushing through Nytex membrane. The cells were collected by centrifugation (1000 rpm 5 minutes) and erythrocytes lysed by addition of 2.5 ml 0.83 % NH_4Cl (pH 7.2). The splenocytes were washed twice with RPMI and the number of viable cells determined by trypan blue exclusion. The cells were then resuspended at $1 \times 10^7/\text{ml}$ (for proliferation studies) or at $2 \times 10^7/\text{ml}$ (for production of cytokines) in RPMI supplemented with 20 % heat inactivated FCS (GibcoBRL) to give a final concentration of 10 %.

Reagents:

Ammonium chloride solution.

0.14 M NH_4Cl

0.02 M Tris-HCl pH 7.65

make to 1 L with ddH₂O

sterilise by autoclaving and store at 4°C.

2.15.5 Proliferation assay.

Splenocyte proliferation in response to antigen or ConA was measured by ^3H thymidine incorporation. Triplicate 100 μl cultures were set up in 96-well half area flat bottomed plates (CoStar) at a concentration of 5×10^5 cells/well. Cells were stimulated with either adult or mf soluble extract at 10 $\mu\text{g}/\text{ml}$ (2.15.1), electro-eluted MMC-1 antigen at 1 $\mu\text{g}/\text{ml}$ (2.9.6) or ConA at 1 $\mu\text{g}/\text{ml}$. The concentrations of antigen and ConA were determined to be optimal in preliminary experiments. After 48 hours (ConA) or 48 and 72 hours (adult/mf/MMC-1 antigen) culture at 37°C, 5 % CO_2 the cells were pulsed for 16 hours with 0.5 μCi of ^3H thymidine/well (1 mCi/mmol,

Amersham). The cells were harvested and the radioactivity measured in a “Topcount” microplate scintillation counter (Canberra Packard Instrument Company).

2.15.6 Analysis of cytokine production by ELISA.

Spleen cells were cultured at 1×10^7 /ml in duplicate 1 ml cultures in 24 well flat bottom plates (CoStar) in the presence of ConA (5 µg/ml), mf/adult antigen (10 µg/ml) or electro-elution purified MMC-1 antigen (1 µg/ml). After 48 hours at 37°C, 5 %CO₂ the supernatants were collected and stored at -70°C. Levels of IL-2, IL-4, IL-5, IL-10 and IFN-γ were measured by two site ELISA using antibody pairs (PharMingen) as described in [96]. Quantities of cytokines in pg/ml were determined by reference to standards of recombinant cytokines. The limit of detection of each assay was defined as the mean plus three standard deviations of 16 wells containing medium (RPMI/10% FCS) only.

The protocol for the cytokine ELISA is as follows. ELISA plates (Corning Easy-wash) were coated with the appropriate capture antibody in PBS (50 µl/well) at 4°C overnight, then blocked with 10% FCS in PBS/0.05% Tween 20 (150 µl/well) for 45 minutes at 37°C. Doubling dilutions of the cytokine standards were prepared in RPMI/10%FCS. The plate was washed with PBS/0.05% Tween 20 (3 x rinse followed by 2 x 3 minute washes) then the standards and samples were added (50 µl/well) and the plate incubated at room temperature for 2 hours. The plates were washed as before and the biotinylated detecting antibody diluted in 1 % BSA in PBS/0.05% Tween 20 was added (50 µl/well) and the plates incubated at room temperature for 1 hour. The plates were washed as before. Streptavidin peroxidase

(Serotec) diluted 1/1000 in PBS/0.05% Tween 20 containing 1 % BSA was added (75 µl/well) and the plate incubated at room temperature for 1 hour. The plate was washed as before then TMB peroxidase substrate (KPL Biotechnology) was added (100 µl/well). After 15 minutes at room temperature the plates were read at 620nm in a Dynatech MR5000 automated ELISA reader.

Reagents:

PBS

137 mM NaCl
8.1 mM Na₂HPO₄
2.7 mM KCl
1.47 mM KH₂PO₄

in ddH₂O, sterilise by autoclaving, store at room temperature.

PBS/0.05% Tween 20

500 µl of Tween 20 was added
per litre of sterile PBS.

2.15.7 IgG responses to MMC-1

To test for the presence of MMC-1 reactive IgG molecules in antisera the following ELISA test was used. ELISA plates (Corning Easy-wash) were coated with 100 µl of either MBP-MMC-1 fusion protein or with MBP2* protein (wild type MBP, New England Biolabs) both at 250 ng/well (in carbonate buffer) at 4°C overnight. The samples were then blocked with 5 % BSA in PBS Tween 20, 200 µl/well for 30 minutes at 37°C. The plate was washed with PBS/0.05% Tween 20 (3 x rinse followed by 3 x 3 minute washes). Serum samples were then diluted 1/100 in PBS/Tween 20 and added to the plates (100 µl/well) for 90 minutes at room temperature. The plates were washed as before and then a goat anti-mouse IgG horseradish peroxidase conjugate (Bio-Rad) diluted 1/1500 in PBS/Tween 20 was added and the plate incubated for 60 minutes at room temperature. The plate was washed as before then a TMB peroxidase substrate (KPL Biotechnology) was added

(100µl/well). After 2 and 4 minutes the plates were read at 620 nm in a Dynatech MR5000 automated ELISA reader. The results were recorded for individual mice and the reactivity of MMC-1 calculated by subtracting the MBP background from the corresponding results.

2.15.8 Human IgG responses to MMC-1

To determine if a humoral response to MMC-1 could be detected in human sera from individuals infected with *B. malayi* ELISA experiments were conducted in collaboration with Dr Xingxing Zang (University of Edinburgh). Serum from subjects characterised as either, asymptomatic mf-, mf + or European normal controls were tested for the presence of MMC-1 specific IgG subclasses. The MBP-MMC-1 fusion protein was used to coat ELISA plates (200 ng/well diluted in 0.06 M carbonate buffer) each serum was reacted in duplicate at a 1/100 dilution (diluted in PBS/0.05% Tween 20). Each serum was also reacted against MBP alone (200 ng/well). Bound antibody was detected using isotype-specific mouse monoclonal antibody (anti-IgG1 1/4000, anti-IgG2 1/2000, anti-IgG3 1/1000 and anti-IgG4 1/4000 diluted in PBS/0.05 % Tween 20, SkyBio). Bound IgG was recognised by peroxidase-conjugated rabbit anti-mouse Ig (1/1500) and developed using ABTS substrate (KPL Biotechnology). The plates were read after 2 minutes at 405nm.

Table 2.1 Oligonucleotide primers.

SL1	5' GCCGGAATTCGGTTTAATTACCCAAGTTTGAG 3'
oligo (dT)	5' GCCGCTCGAGTTTTTTTTTTTTTTTTTTT 3'
adapted oligo (dT)	5' GTCAGATCTACGCGTCGACCTCGAGTTTTTTTTTTTTTTTTTTT 3'
adapter	5' GTCAGATCTACGCGTCGACCTCGAG 3'
T3	5' AATTAACCCTCACTAAAGGG 3'
T7	5' TAATACGACTCACTATAGGG 3'
<i>mmc</i> -1F1	5' GCATTTAGTGCAACCATCGCTGATG 3'
<i>mmc</i> -1R1	5' ACGTCGAAAGAGTAAACCAGCATCG 3'
<i>mmc</i> -1R3	5' CGTTCCTTGCGATCCTGGG 3'
<i>mmc</i> -1gsp1	5' CAACAACAGCAACATCCTTC 3'
<i>mmc</i> -1gsp2	5' TTTGCGATCCTGGGTCTCAG 3'
<i>mmc</i> -1gsp3	5' TCATCAGCGATGGTTGCAC 3'
5' RACE AAP	5' GGCCACGCGTCGACTAGTACGGGIIIGGGIIIGGGIIG 3'
5' RACE AUAP	5' ' GGCCACGCGTCGACAGTAC 3'
MMC-1ExF1	5' GCCGGGATCCATGAAATATT 3'
MMC-1ExR1	5' GCCGCTGCAGTCAACAACAG 3'
<i>vmc</i> -2F1	5' GCACTTACTGCCCAAATGTCAGTTG 3'
<i>vmc</i> -2R1	5' ACCACTGTGCAAATAGTATCCAATG 3'
<i>vmc</i> -2gsp1	5' GGAAGTTGAAGCAGTACGACCGTGGG 3'
β -tubA	5' AATATGTGCCACGAGCAGTC 3'
β -tubB	5' CGGATACTCCTCACGAATTT 3'
T3 [*]	5' AATTAACCCTCACTAAAG 3'
T7 [*]	5' TAATACGACTCACTATAGGG 3'
M13rev [*]	5' CAGGAAACAGCTATGAC 3'
M13 [*] (-40)	5' GTTTTCCCAGTCACGAC 3'

* Fluorescent (IRD800 labelled) primers corresponding to these sequences were used for LI-COR automated sequencing. Primers were synthesized by MWG.

CHAPTER THREE

3.1 Introduction.

The aim of this project was to isolate and characterise genes that are differentially expressed by the mf of *Brugia pahangi* when in the mosquito and mammalian hosts. The approach adopted was a differential screening of a mammalian-derived cDNA library using cDNA probes prepared from two populations of mf, i.e. mosquito and mammalian derived. A similar method has previously been used in this laboratory for the isolation of cDNA clones differentially expressed in the mammalian-derived L3 compared to the vector-derived L3 [76, 79]. The experiments reported in this chapter involved the preparation and screening of a mammalian-derived mf cDNA library. For the purpose of carrying out differential hybridisation, two populations of cDNA are required. In this case, one from mammalian-derived mf and the second from vector-derived mf. Ideally to obtain vector-derived mf for the production of cDNA probes a mass dissection of infected mosquitoes would be undertaken. However this is impractical due to the small size of the mf, approximately 280 μm X 5-6 μm (24 hours post infection) [9], and the intramuscular nature of infection within the mosquito flight muscles. This constraint on the availability of *in vivo* mosquito-derived mf material for RNA extraction and consequent cDNA production, necessitates an alternative approach. The approach described in this chapter was to culture mf *in vitro*, using a system that might mimic *in vivo* events, and which might stimulate gene expression comparable to that of mf in the insect vector. As the vector-derived mf were produced by *in vitro* culture, the mammalian-derived mf were treated in a comparable way. This was considered important so that extraneous variables introduced by additional purification and culture steps would be equivalent in each of the two culture systems. Any differences

observed in gene expression between the two populations could therefore be more confidently attributed to differences in the culture systems.

As discussed in Chapter One, *B. pahangi* mf in the bloodstream of the definitive mammalian host are enveloped in the microfilarial sheath. Following ingestion with the bloodmeal the mf undergo a migration through the mosquito. The mf penetrate the mosquito midgut and pass through the haemocoel before migrating to the site of development, primarily the thoracic muscles, where they initiate development [8]. At some point during this migration the microfilarial sheath is lost [171]. As the loss of the sheath is a pre-requisite for development of the mf, attempts to mimic the *in vivo* development of the mf require that the sheath be artificially removed for the culture experiments. To this end the mf were artificially exsheathed and then allowed to migrate through a pad of solidified low melting temperature agarose; this had the dual purpose of retaining any mf damaged during exsheathment and mimicking the migration through the mosquito midgut. Exsheathed mf were then cultured in a number of different media to ascertain the optimal conditions for survival *in vitro*. From these worms RNA was isolated and used in subsequent cDNA preparation to produce the probes that were used for the differential screening of the mammalian mf cDNA library.

Initially a *B. malayi* mf cDNA library obtained from the Filarial Genome Consortium was utilised. The library was differentially screened with complex cDNA probes produced by RT-PCR of either mammalian or vector-derived *B. pahangi* RNA (as described in section 2.5). However as no differentially expressed cDNAs were isolated in the differential screen, it was deemed necessary to screen a new mf library produced under controlled conditions.

3.2 Results.

3.2.1 Artificial exsheathment of mf.

It has been shown previously that the exposure of the mf of *B. pahangi* to proteases or concentrations of calcium greater than 10 mM, stimulates exsheathment *in vitro* [172]. In an attempt to repeat these experiments, two proteolytic enzymes, Pronase, (Sigma type XIV) from *Streptomyces griseus* and Subtilisin (Sigma type VII), were compared for their capacity to exsheath *B. pahangi* mf *in vitro*.

Using a 24 well tissue culture plate, a series of doubling dilutions ranging from 500 µg/ml to 1.95 µg/ml of each enzyme was prepared by serial dilution of a stock solution with HBSS. To each well approximately 2.5×10^4 mf, purified from an infected jird, and suspended in HBSS were added. The process of exsheathment was monitored at set time points using phase-contrast microscopy. In the preliminary experiments the ability of Pronase to promote exsheathment was observed to be greater than that of Subtilisin. Mf that are destined to exsheath adhere to the plastic substrate and exhibit a thrashing movement until the sheath ruptures and the exsheathed mf are released. After five minutes in all concentrations of Pronase solution, mf showed the characteristic adherence to the substrate. After a ten minute incubation in Pronase (500 µg/ml) a large number of the worms were exsheathed and were free-swimming; in comparison, even after one hour of incubation in the highest concentration of Subtilisin (500 µg/ml), no exsheathment was observed, although the mf were still motile. Following this initial experiment, Pronase was selected for further experimentation to determine the optimal conditions of concentration and incubation time for exsheathment.

The percentage exsheathment of mf was compared after 5, 10, 15 and 40 minutes using three concentrations of Pronase, 2, 1 and 0.5 mg/ml diluted in HBSS. At the set time points 25 µl of the mf suspension was spotted onto a microscope slide and the mf killed by passing over a flame. After observation by phase-contrast microscopy the percentage of exsheathed mf was calculated. The results are shown graphically in Figure 3.1. From these experiments a protocol of exposure to 1 mg/ml Pronase for 10 minutes was considered optimal. The exsheathment step was followed by two washes in HBSS + 10% FCS, and a final wash in HBSS with no added FCS. The addition of FCS to the wash solutions was intended to "mop-up" any residual enzyme. The full details of the exsheathment protocol are described in section 2.4.1.

3.2.2 Agarose pad purification of exsheathed mf.

In an attempt to mimic the migration of the mf from the bloodmeal to the site of development the mf were purified through an agarose pad. This technique is a modification of the process of purification first described by Greene and Schiller (1979) [165]. The technique is described in detail in section 2.4.1. Mf migrate through an agarose pad and can be collected from the surface medium (Figures 3.2 and 3.3). As well as mimicking the migration of the mf, this technique requires an active effort from the mf and therefore has the advantage of selecting a homogeneous population of mf, leaving the weaker and less fit mf in the agarose pad. A disadvantage of the technique is that a proportion of the mf are lost. In order to quantify the numbers of mf that were being retained in the agarose pad, counts of mf were taken at sequential points during the process of purification. Mf counts were taken, prior to, and following exsheathment (points A and B of Figure 3.3,

Figure 3.1. Percentage exsheathment of *B. pahangi* mf induced by incubation with Pronase enzyme.

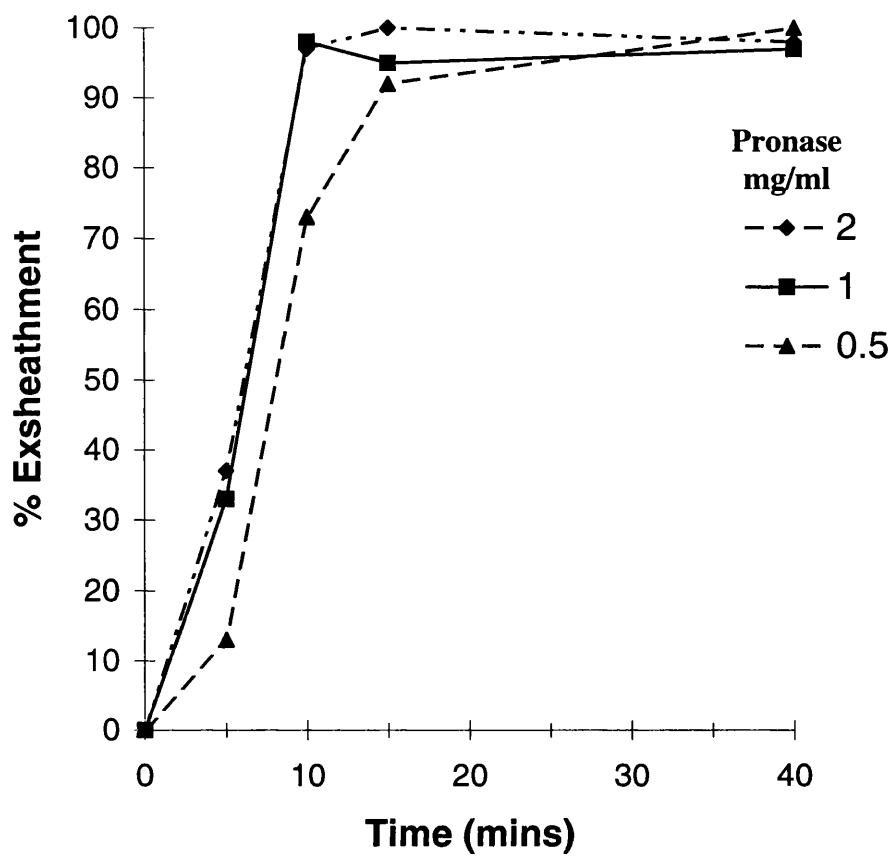


Figure 3.1
Approximately 2.5×10^4 mf were incubated at room temperature in either 2, 1 or 0.5 mg/ml Pronase in HBSS. The percentage exsheathment after 5, 10 15 and 40 minutes was determined by phase-contrast microscopy.

Figure 3.2. Agarose pad purification of microfilariae.

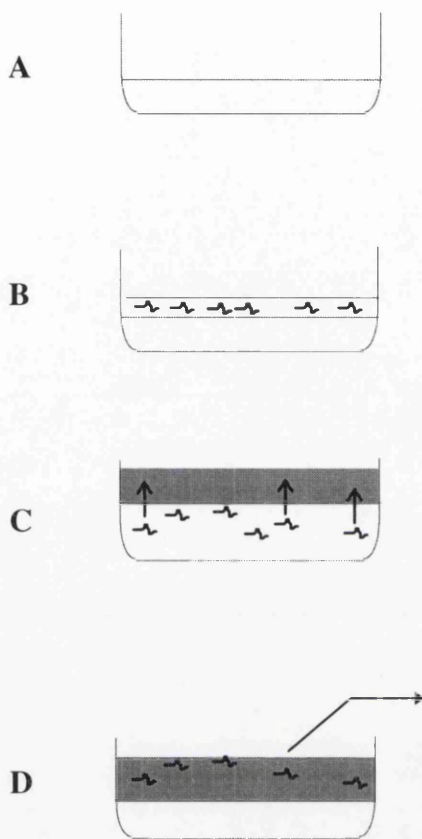
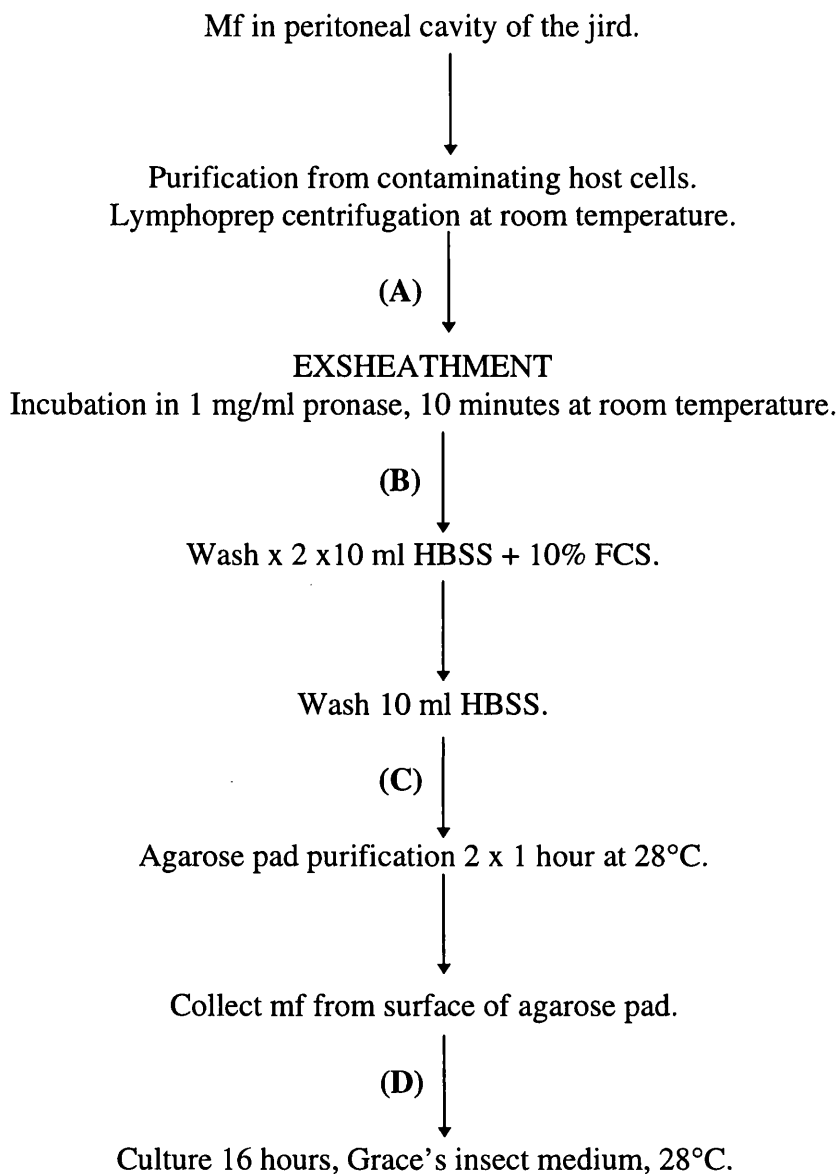


Figure 3.2

Following the exsheathment the mf were purified by migration through a low melting point agarose pad. **A)** 1 ml of molten 2% low melting point agarose is placed in a 35 mm petri dish. **B)** 1 ml of mf in Grace's insect tissue culture medium is added and mixed. **C)** The 1 % agarose mixture is overlayed with fresh Grace's medium and incubated at 28°C for 1 hour. The mf migrate through the agarose pad into the Grace's medium. **D)** The medium containing the mf is removed. For best returns of mf steps **C** and **D** were repeated.

Figure 3.3 Flow diagram of protocol for production of vector-derived mf.



respectively) and prior to, and following agarose pad purification (points C and D of Figure 3.3, respectively). Mean numbers of mf were calculated from duplicate 25 µl samples taken at each of the time points. The results of the total number of mf at each time point are shown in Table 3.1. The total percentage recovery of mf through the agarose pad in this experiment $((D_i + D_{ii}) \div C) \times 100$ is 98.2%. In contrast the percentage recovery after exsheathment $(B \div A) \times 100$ is 50.9%, and after the washing procedures $(C \div B) \times 100$ is 34.5%. The loss at these stages is likely to be due to mf adhering to the plastic tubes during the exsheathment and washing stages of the protocol. The accumulative effect of these losses means that only 17.2% of the mf purified from the jird are available for the production of vector-derived mf cDNA. Although this places a large demand on the number of mf need to produce cDNA, it was considered important to culture the mf in conditions that were thought to mimic *in vivo* conditions.

3.2.3 Comparison of different media for the culture of *B. pahangi* mf.

The aim of these experiments was to determine optimal conditions for the culture of exsheathed mf. Initially the survival of exsheathed mf was compared in a mammalian medium, minimal essential medium (MEM) plus 10% FCS at 37°C or in the vector medium, Grace's insect tissue culture medium at 28°C. The mf were cultured in 2ml of appropriate culture media at a concentration of approximately 7×10^3 mf ml⁻¹, in Nunclon flat-sided tissue culture tubes. The survival and behavior of the mf after 16 and 24 hours was compared between the two media, using phase contrast microscopy. After 24 hours the mf in both the vector and mammalian culture

Table 3.1 Recovery of mf after exsheathment and agarose pad purification.

Time Point.	Mean number of mf.	Percentage of pre- exsheathment. (Recovery)
A. Pre-exsheathment.	139000	100
B. Post-exsheathment.	70750	50.9
C. Pre-purification.	24400	17.6
Di Post purification (1 hour)	14500	10.4
DII. Post-purification. (2 hours)	9450	6.8

Table 3.1
Duplicate counts of mf were taken at stages during the exsheathment and purification of *in vitro* vector-derived mf. Percentage recovery at each of the time points was calculated by comparison to the initial number of worms at time point A.

systems were motile suggesting that the process of exsheathment did not affect the viability of the mf.

Following these preliminary experiments a number of different culture media, developed for the culture of insect cells, were compared to determine the best medium for vector-derived mf culture. The following media were compared: Grace's insect medium, Grace's insect medium supplemented (contains 3300 mg/L lactalbumin hydrolysate), IPL-41 insect medium, SF-900 II serum free insect medium, Schneider's *Drosophila* medium (revised) and TC-100 insect medium (all Gibco Life Technologies). Using 24 well culture plates each medium was tested under the following conditions: medium alone; medium + 10% FCS; medium + 0.25% HEPES; medium + 10% FCS + 0.25% HEPES. FCS was added to assess differences in mf survival and behaviour in simple versus more complex medium. HEPES was added to buffer the pH of the medium to approximately pH 7. A total of 1×10^3 mf were added to each of the media and the plates were observed at 16, 20 and 24 hours. After 24 hours the majority of mf in all conditions were motile and there was no visible signs of contamination. The addition of either 0.25% HEPES or 10% FCS to the cultures did not significantly affect the survival of the mf and as it was considered desirable to keep the culture system as simple as possible these were excluded from the final culture system. Two of the insect media gave particularly interesting results, Grace's insect medium (unsupplemented) and IPL-41 insect medium. The viability of the mf (as determined by motility) was optimal in both of these media. However, observation showed that the mf behaved quite differently in each media. The mf in Grace's medium showed relatively little motility but remained elongate with movement confined to the anterior and posterior ends, whilst the mf in

IPL-41 exhibited a thrashing movement along the whole length of the body. These findings are interesting as the behaviour of mf freshly harvested from infected jirds or cultured in mammalian medium (MEM + 10% FCS, 37°C) resembles that of those in IPL-41. In contrast, the behaviour of mf in Grace's insect medium resembles that of mf developing in infected mosquitoes [9].

Following these experiments, the final culture conditions selected for vector-derived mf were as follows: exsheathment, agarose pad purification and incubation at 28°C + 5%CO₂ for 16 hours in Grace's insect medium (unsupplemented). An overview of the procedure for the production of vector-derived mf is shown in Figure 3.3.

3.2.4 Comparison of cDNA produced from mammalian-derived and vector-derived mf.

Total RNA was isolated using a TRIzol (Gibco, Life Technologies) based method (section 2.2.15) from mf cultured in either mammalian or vector-like conditions. First strand cDNA was synthesised using superscript reverse transcriptase, (Gibco, Life Technologies) and an adapted oligo (dT) primer (section 2.2.18). cDNAs were amplified using one primer that anneals to the adapter region of the adapted oligo (dT), and a SL1 primer that binds to the conserved nematode SL1 *trans*-spliced sequence. It was anticipated that PCR amplification would result in a complex mix of cDNAs that would represent the mRNAs present in the cells of mf when cultured under mammalian and vector-like conditions.

Figure 3.4. Electrophoresis of PCR-amplified cDNA produced from mf cultured under vector-like and mammalian-like conditions.

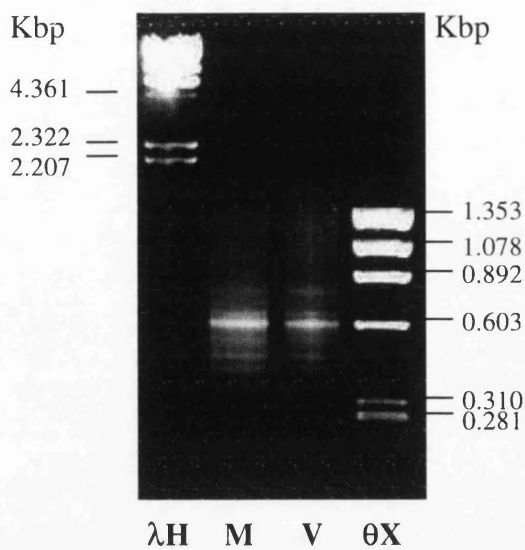


Figure 3.4

Approximately 2 µg of total RNA was isolated by TRIzol treatment, from approximately 1×10^6 mf mass cultured under either mammalian or vector-like conditions. First strand cDNA was generated with adapted oligo (dT) and the resulting first strand cDNA was PCR amplified for 35 cycles as follows: 94°C/1min, 55°C/1min 72°C/1min, followed by a final extension of 10 minutes at 72°C. 5 µl of resulting PCR products were run on a 1 % agarose gel.

λH = *Hind* III digested λ DNA molecular weight markers.

θX = *Hae* III digested θX174 DNA molecular weight markers.

M = cDNA of mf cultured in mammalian like conditions (MEM + 10% FCS, 37°C).

V = cDNA of mf cultured in vector-like conditions (mf artificially exsheathed, Graces insect tissue medium, 28°C).

By visual comparison of the two cDNA populations electrophoresed on an ethidium bromide stained agarose gel, no clear differences could be observed (see Figure 3.4) The lack of gross differences between the two cDNA populations, which would be seen as more prominent bands in one sample compared to the other, excluded the possibility of cloning cDNAs by direct excision from an agarose gel. As the differences between the two cDNAs populations was below this level of detection a more sensitive method would need to be employed. To this end the two cDNA populations were used as the probes to hybridise to a mammalian-derived mf cDNA library, as discussed below.

3.3 Screening of a *B. malayi* mf cDNA library.

A conventional mf cDNA library (SAW94LS-BmMf) was supplied by the filarial genome project resource centre at Smith College, Northampton, MA 01063, USA. A total of 1×10^4 pfu was plated on five 130mm petri dishes and grown overnight at 37°C. Phage were transferred to duplicate nylon filters and the filters probed using ^{32}P radiolabeled cDNAs produced from vector-derived or mammalian-derived *B. pahangi* mf, as described in section 2.5.2. The specific activity of the probes was similar as determined by calculating the incorporated cpm. The filters were washed at high stringency (65°C washed to 0.1 X SSC). and exposed to autoradiographic film overnight at -70°C. From this primary screen 37 plaques hybridised more strongly to the mammalian probe. Initially the ten most strongly hybridising phage were picked, titrated and a secondary screen at low density (2×10^2 pfu) was performed. When the resulting autoradiographs were examined no

differential hybridisation was observed. This secondary screen was repeated but again no differential hybridisation was seen. As the secondary screen did not produce any differentially hybridising cDNAs (for possible reasons discussed in section 3.5), a new *B. pahangi* mf cDNA library was prepared.

3.4 Construction of a *B. pahangi* cDNA library.

A *B. pahangi* cDNA library was constructed using mf collected from the peritoneal cavity of jirds, *Meriones unguiculatus*, infected at least three months previously with 250 infective L3. The mf were purified from contaminating host cells, washed and cultured in mammalian-like conditions (37°C MEM + 10% FCS) for four hours prior to snap freezing in liquid nitrogen, as described in detail in section 2.3.1. All manipulations were optimised to avoid prolonged exposure to ambient conditions. Due to the small amounts of starting material available the library was constructed by reverse transcriptase PCR (RT-PCR) [154], using the nematode spliced leader (SL1) and an adapted (dT) primer. An adapter primer rather than oligo (dT) was utilised in order to try and reduce internal priming at adenine rich regions and therefore increase the chance of amplifying full-length cDNAs. The use of the adapter primer also allows the annealing temperature of the PCR to be raised, further increasing the specificity of priming. In order to optimise the quality of the library, total RNA was extracted from three different cultures of mammalian-derived mf and this was used in separate RT reactions with adapted oligo (dT) primer. Six separate 10 cycle PCRs were conducted utilising the first strand cDNA as a template. Products from more than one PCR were mixed in order to minimise any preferential amplification that may have occurred in one of the reactions, and also to reduce the

frequency of PCR errors. The PCR products were then size separated using a Sepharose 400 column (Pharmacia), and cDNAs of a size greater than 400bp were collected into 1 X PCR buffer (Perkin Elmer). An aliquot of this fraction was then used in a 20 cycle PCR using the same conditions as before. The resulting cDNA pooled from the various PCRs was again size separated, digested with *Eco* RI and *Xho* I and ligated into λ -ZapII (section 2.3.2) The resulting primary library was amplified (section 2.3.4) and stored at -70°C. For an overview of the two-step PCR protocol see Figure 3.5.

3.4.1 Characterisation of the *B. pahangi* mf cDNA library.

The primary library had a titre of 3.3×10^5 pfu and contained 94% recombinants, as determined by α -complementation (section 2.3.3). The range of insert sizes was determined by PCR on fifty randomly picked well-separated plaques. PCR was performed using the primers corresponding to the T7 and T3 vector sequences that flank the multiple cloning site of the Bluescript plasmid. After amplification, the products were electrophoresed through an agarose gel containing ethidium bromide. By subtracting the size of T3-T7 in a non-recombinant phagemid (167 bp) from that of the individual recombinants, the insert size could be calculated. The sizes ranged from 200-950bp with an average of approximately 500 bp (Figure 3.6).

In an attempt to estimate the percentage of clones that might be differentially expressed and thus to estimate how many clones should be screened, 940 plaques were picked and plated in an ordered array and replica filters were produced using a

Figure 3.5 Two-step PCR protocol for construction of mammalian-mf cDNA library.

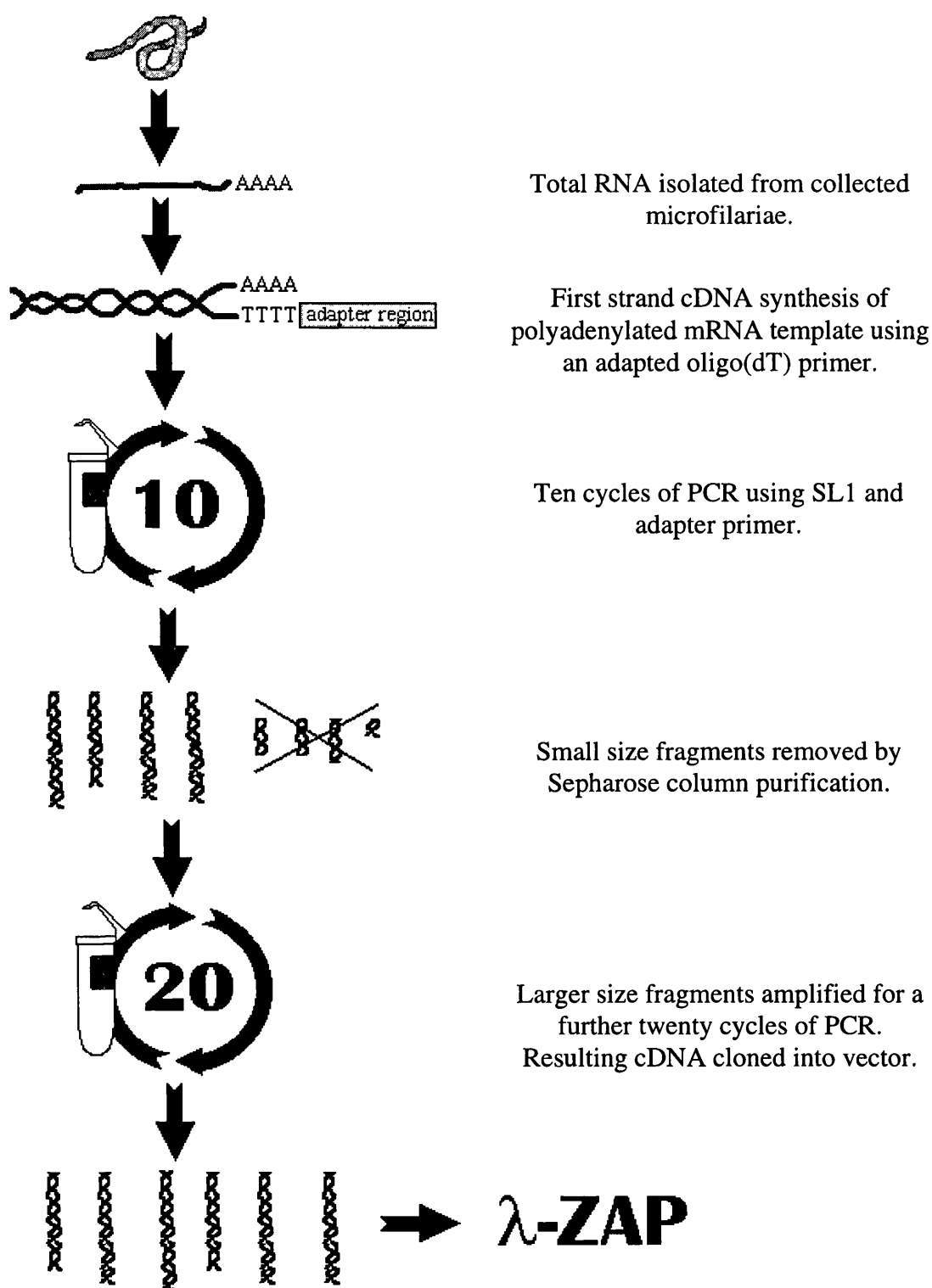


Figure 3.6 Determination of library insert size by PCR.

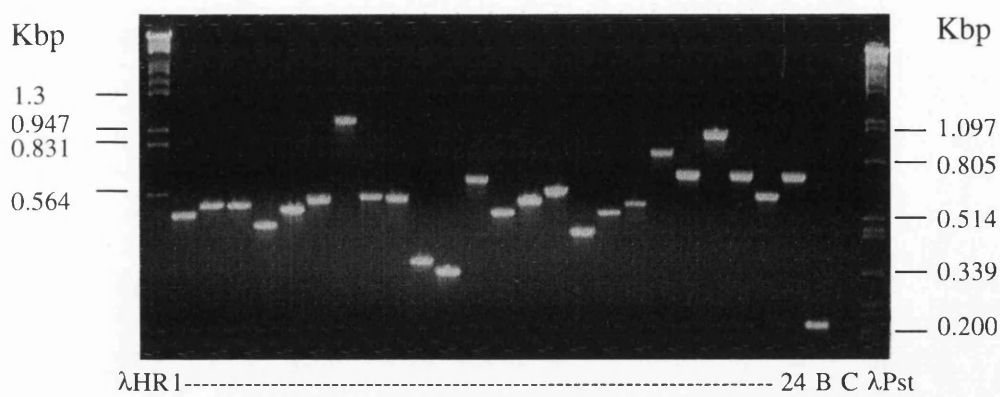


Figure 3.6
10 µl of each phage suspended in SM buffer were amplified using T3 and T7 primers which flank the cloning site of the Bluescript plasmid. Amplification for 30 cycles as follows: 94°C/1min, 60°C/1min 72°C/1min.

λHR = *Eco* RI and *Hind* III digested λ DNA molecular size markers.
1-24 = 10 µl of PCR products of individual well separated plaques from *B. pahangi* mammalian mf cDNA library.
B = 10 µl of non-recombinant Bluescript phagemid PCR product.
C = no template PCR control.
λPst = *Pst* I digested λ DNA molecular size markers.

mechanical replicator [173]. Duplicate filters were probed with vector-derived and mammalian-derived mf cDNAs as described previously. The filters were washed to high stringency (65°C washed to 0.1 X SSC 0.1 % SDS) and exposed to autoradiographic film overnight at -70°C. In this test experiment all plaques hybridised with equal intensity to both of the probes. This experiment indicated that an excess of 940 plaques would need to be screened to isolate any differentially expressed genes.

3.4.2 Differential screening of the *B. pahangi* cDNA library.

The primary library was used in this differential screen as an amplified library may not be truly representative of the starting RNA sample, due to the possibility of greater amplification of some plaques during this procedure. 2×10^4 pfu of the primary library was plated on ten NZY agar plates with *E. coli* XL1-Blue MRF'. The bacteriophage were grown overnight at 37°C then transferred to duplicate nitrocellulose filters, prior to fixation and probing [153]. The filters were probed, at high stringency (65°C, washed to 0.1x SSC, 0.1 SDS) with either mammalian-derived or vector-derived mf cDNAs, produced as described previously, then exposed to autoradiographic film. Plaques containing DNA that hybridised differentially to either probe were selected for further rounds of screening to confirm initial hybridisation results, Figure 3.7 shows an example of a differential screen in which duplicate filters were probed with either mammalian or vector derived mf probes.

Although the initial aim was to isolate genes up-regulated in the mammalian-derived mf, a number of cDNAs which hybridised more strongly to the vector-mf probe were observed. These were also selected for further analysis. From the initial

Figure 3.7 An example of a differentially hybridising cDNA screen.

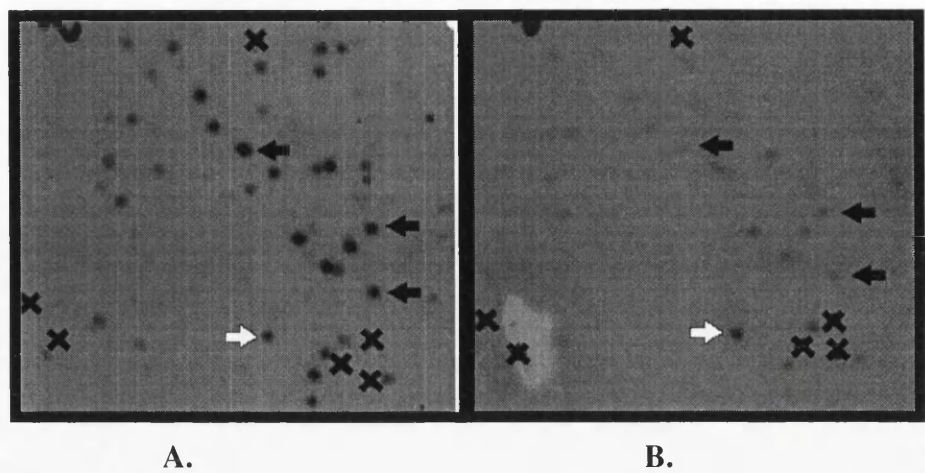


Figure 3.7

5×10^2 pfu from the primary screen were plated on 130 mm petri dishes and duplicate phage filter lifts were prepared. Filters were hybridised with $^{32}\text{P}\alpha\text{-dCTP}$ labelled cDNA probes at 65°C and washed to 0.1 X SSC, 0.1% SDS.

A = Filter probed with the mammalian-derived mf cDNA.

B = Duplicate filter probed with vector derived mf cDNA.

X = marks for orientation of duplicate filters.

Closed arrows show examples of corresponding plaques that are differentially hybridising. Open arrows show an example of a plaque hybridising equally to each probe.

primary screen of 2×10^4 pfu, eighty one plaques which hybridised differentially were selected for secondary analysis, of which 67 hybridised more strongly to the mammalian-derived mf cDNA probe. The putative positive plaques were cored from the agar and resuspended in SM buffer, as described in section 2.5.2. These were titrated and subjected to secondary screening at a density of 5×10^2 pfu on a 130 mm petri dish, under the same stringency conditions as stated for the primary screen. The secondary screen resulted in 28 plaques which strongly hybridised to the mammalian-derived mf probe and seven which hybridised more strongly to the vector-derived mf probe. As the secondary screen was conducted at low density, well separated positively hybridising plaques could be picked for confirmation of hybridisation pattern by tertiary analysis using a reverse northern technique.

The eighty-one differentially hybridising plaques were also plated in an ordered array using the mechanical replicator, and a test hybridisation using a probe specific for *B. pahangi* heat shock protein 90 cDNA (*hsp90*) was conducted. *hsp90* was chosen as this gene has previously been shown to be up-regulated in mammalian-derived mf (A. Cockroft, University of Glasgow, personal communication). One of the duplicate ordered array filters was hybridised under high stringency conditions to a gel purified fragment of the *hsp90* gene produced by PCR using SL1 and an internal gene specific primer. After washing to high stringency ($0.1 \times$ SSC 0.1% SDS at 65°C) the filter was exposed to autoradiographic film. Figure 3.8 shows the developed autoradiograph in which the probe can be seen clearly hybridising to a single plaque. The low level of hybridisation to all other plaques may be due to the use of SL1 in the production of the probe, as all cDNAs isolated should

Figure 3.8 Hybridisation of primary screen positives with *hsp90*.

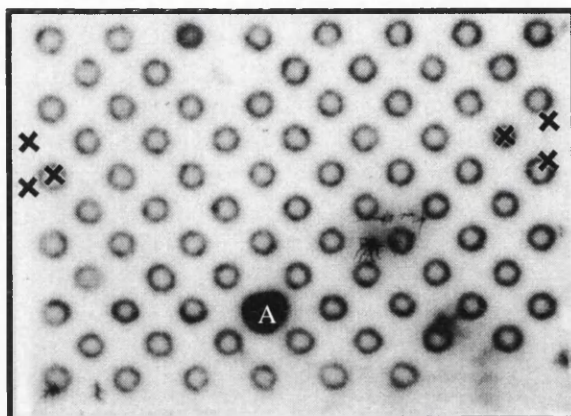


Figure 3.8

Eighty-one positives from the primary screen were plated in an ordered array using a mechanical replicator. One duplicate filter was hybridised with radiolabelled *hsp90* gene-specific probe. The filter was washed at 65°C to 0.1 X SSC, 0.1% SDS, and exposed to X-ray film for 7 days.

X = orientation marks

A = Positively hybridising *hsp90* cDNA.

contain the SL1 sequence due to the technique employed in producing the cDNAs for the library.

3.4.3 Confirmation of differential expression by reverse northern.

In order to confirm the differential expression of the putative positives remaining after the secondary screen a reverse northern method was utilised. Northern analysis [159] involves the separation of RNA through a gel matrix and subsequent transfer to a nylon membrane for probing with a DNA probe. In the reverse northern technique DNA is separated, transferred and then hybridised with cDNA which is representative of the RNA of interest.

The method used here was a modification of the technique described by Fryxell and Meyerowitz (1987) [174], as the cDNA of interest is amplified by PCR rather than digested from a plasmid vector. Full details of the technique are given in section 2.5.2. Briefly, differentially hybridising phage were "tooth-picked" from agar plates, liberated into SM buffer and allowed to dissociate. An aliquot of this was then used as the template in a PCR reaction using T3 and T7 primer sequences that flank the cloning site of the phagemid vector. Equal quantities of PCR products were diluted (1:100) and analysed in duplicate in two halves of an agarose gel, following which the DNA was transferred to a nylon membrane using standard methods. By subsequently bisecting the membrane, two identical blots were produced which were differentially screened using the same probes as before. One of the major disadvantages of screening phage DNA immobilised on nitrocellulose is the assumption that equal amounts of template DNA adhere to each of the duplicate plaque lifts. By normalising the template DNA on the membranes by the reverse northern method the pattern of hybridisation observed is accepted with greater

Figure 3.9 Reverse northern confirmation of differentially expressing cDNAs.

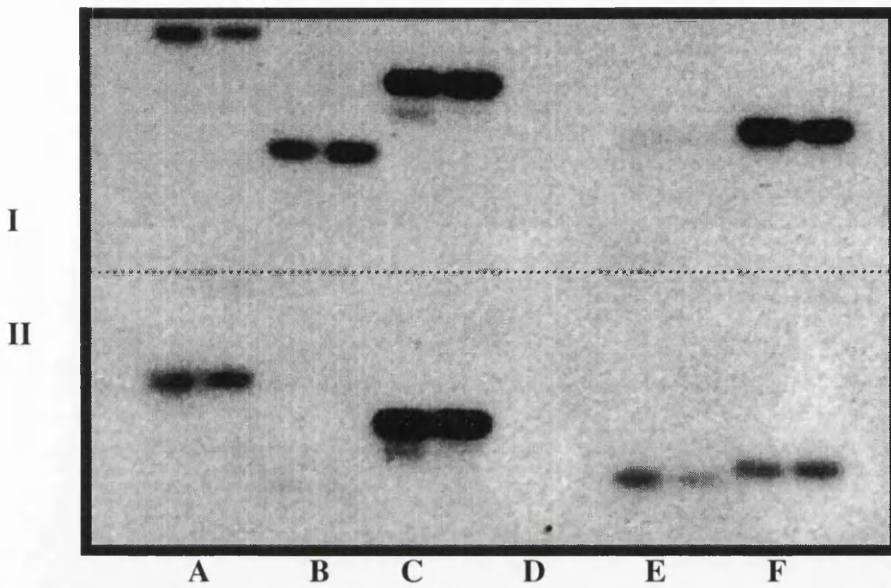


Figure 3.9

10 μ l of phage suspended in SM buffer were amplified by PCR using T3 and T7 primers. Amplified products were run in duplicate on a 2 % agarose gel and DNA transferred to nylon membrane. Resulting reverse northern divided into two identical filters and hybridised to either mammalian-derived or vector-derived cDNAs.

Panel I = Mammalian-derived mf cDNA probe.

Panel II = Vector-derived mf cDNA probe.

A/C = represent constitutively expressed mRNAs.

B/F = mRNAs up-regulated in mammalian-derived mf.

D = cDNA weakly hybridised, would be exposed for longer.

E = mRNA up-regulated in the vector-derived mf.

confidence. Figure 3.9 shows an example of results from a reverse northern experiment. After this tertiary screen twenty-one cDNAs were confirmed to be differentially hybridising, of which seventeen hybridised more strongly to the mammalian-derived mf cDNA, and four to the vector-derived mf cDNA.

3.4.4 Analysis of differentially expressed cDNAs.

The twenty-one cDNAs were *in vivo* excised to liberate the recombinant Bluescript phagemid, (section 2.5.3) and initially sequenced on one strand using the M13 reverse sequencing primer (section 2.2.20). The resulting sequence data was compared by a BLAST search [124] to Genbank and EMBL nucleotide sequence databases. A summary of the results of sequence homologies are shown in Table 3.2. Table 3.2 also shows the abundance of homologous *B. malayi* ESTs throughout the life-cycle. The isolation of 21 differentially hybridising cDNAs from a screen of 2×10^4 pfu, corresponds to approximately 1 in 1000 cDNAs from the library representing genes differentially expressed in the mf stage of *B. pahangi*. The 21 differentially hybridising cDNAs represented nine differentially expressed genes. Five of the genes isolated were ribosomal protein genes, one was *B. pahangi hsp90* and three were novel genes.

3.4.5 Selection of genes for further study.

From the twenty-one differentially expressed cDNAs isolated in this study two were initially selected for further analysis recDNA1 and recDNA76. The cDNAs were renamed in accordance with the suggested nomenclature for *Brugia* genes [175], recDNA1 was renamed Bp-*mmc*-1 (*B. pahangi*-mammalian-derived mf

Table 3.2 Clones isolated from *B. pahangi* mammalian-derived mf library.

Clone	Acession Number	Homology	Expression pattern	No. of times isolated	Size (bp)
recDNA35	AJ277715	A1893523	M.mf	4	464
recDNA20	AJ277714	<i>Bm-rpl-15</i>	M.mf	1	356
recDNA57	AJ277716	<i>Bm-rpl-36</i>	M.mf	2	424
<i>Bp-hsp90</i>	AJ005784	<i>Bp-hsp90</i>	M.mf	1	N/A
recDNA1	X95664	BpcDNA5	M.mf	9	297
<i>Bp-EE4</i>	X91066	<i>Bm-rpp-1</i>	V.mf	1	549
recDNA53	AJ277717	<i>Bm-rpl-38</i>	V.mf	1	362
recDNA13	AJ277713	Novel	V.mf	1	147
recDNA76	AJ277712	Novel	V.mf	1	299

<i>Bm</i> cluster BMC	All ests	Mf	L2	L3	L4	Adult male	Adult female
00124	17	0	1	4	4	2	6
00151	37	9	9	3	4	6	6
00060	47	3	11	5	21	5	2
05057	8	0	0	4	0	1	1
00480	3	3	0	0	0	0	0
00166	70	5	6	14	24	12	9
00157	28	0	4	5	15	1	3
--							
--							

Table 3.2

The left hand portion of the table shows the expression and abundance of the cDNAs isolated from the *B. pahangi* library.

The right hand portion of the table shows the abundance of corresponding ESTs from the *B. malayi* cluster database.

rpl = large subunit ribosomal protein.

M.mf mammalian-derived mf up-regulated.

V.mf vector-derived mf up-regulated.

Bm = *B. malayi*.

cDNA-1) and recDNA76 was renamed Bp-*vmc-2* (*B. pahangi*-vector-derived mf cDNA-2). *mmc-1* represented the most abundant differentially expressed cDNA within the library; it was found nine times during the differential screen of 2×10^4 plaques. *mmc-1* was first isolated in this laboratory by Dr Fiona Thompson whilst studying the small heat shock proteins of *B. pahangi*, (bpcDNA5, accession number X95664). Then, as now, the clone showed no significant homology to any published sequences, other than to itself. The fact that *mmc-1* does not show significant homology to sequences in the *C. elegans* genomic database, which is now completed, or the EST database, that would more readily produce data on abundant sequences, suggests that this may be a *Brugia*-specific gene. Whether it is specific to filarial nematodes or is present in more divergent species is an interesting question. *mmc-1* was potentially very interesting as it may represent an important gene in the maintenance of the developmental block exhibited by the mf in the mammalian blood stream, or in the mf-specific modulation of the host immune system.

Although the initial aim of this study was to identify genes up-regulated in the mammalian mf, *vmc-2*, was also chosen for further study as relatively little is known of the biology of the parasite when developing in the mosquito. As this gene is up-regulated upon transfer to the vector it may play an interesting role in the process of adaptation to the new host. Upon entering the mosquito host the mf find themselves in a different physico-chemical environment, and must rapidly adapt to these conditions whilst resuming development. Development within the mosquito involves profound morphological and presumably physiological changes, and it is possible that this gene may play a role in this process of adaptation.

3.5 Discussion.

The initial aim of this project was to screen by differential hybridisation a *B. malayi* mf library. The availability of cDNA libraries from the filarial genome consortium offers a tremendous advantage to laboratory researchers as time and materials are more effectively utilised. Unfortunately no differentially hybridising cDNAs were isolated from this library. The fact that hybridisation of the *B. pahangi* probes was observed, but not in a differential manner, suggests that the lack of results is not due to inter-specific differences of probe (*B. pahangi*) and library (*B. malayi*) cDNAs. Studies in this, and other laboratories, have shown a high level of homology between the two species. The lack of isolated cDNAs may relate to the numbers screened. 10,000 pfu were screened and from the initial studies it was likely that only 0.1 % of the pfu represented differentially expressed genes, this would predict that only 10 differentially hybridising plaques would be observed at most. Alternatively the lack of positive results could relate to the conditions of library construction. After discussion with the Filarial Genome Project resource centre it became apparent that infected jirds were purchased from an external source but no information was available as to the conditions under which the mf were handled. In order to exclude these potential variables from this study it was considered essential to produce a cDNA library using mf that were collected and purified under known conditions.

Harvesting and purification of the mf from the jird necessitates some unavoidable exposure to ambient temperature, e.g. during centrifugation steps. Furthermore, the only way that a sufficient number of vector-derived mf could be collected was by exposing them to insect-like conditions *in vitro*. Therefore the mammalian mf used for the construction of the library, were similarly exposed to a

mammalian *in vitro* culture system, in the hope that any differences induced solely by handling mf during culture would be equivalent in the two populations of mf. The need to produce vector and mammalian cDNAs from *in vitro* cultured material, imposed restrictions on their production. The mf had to be cultured in a medium resembling the natural environment of the vector and definitive hosts; therefore standard mammalian and insect tissue culture media were used, and the mf for vector-derived mf culture had to be exsheathed. The method by which exsheathment occurs *in vivo* is still unknown. Exsheathment has been reported to occur in different sites during the migration of the parasite through the mosquito, for example exsheathment of *B. pahangi* mf in *Anopheles quadrimaculatus* occurs in the midgut almost immediately after ingestion [171]. In contrast some reports suggest that penetration of the midgut by the mf ruptures or weakens the sheath to allow exsheathment in the haemocoel, for review see [171] [176]. Because of the lack of evidence as to where the mf exsheath it is difficult to speculate on the factors which may be involved in the process of exsheathment. Regardless of the method, the actual process of exsheathment or subsequent stimulation of the cuticle minus the sheath may provide an important cue for development, and as such it was considered important to exsheath the mf prior to culture. Devaney and Howells (1979) showed that the exposure of *B. pahangi*, *B. malayi*, *W. bancrofti* and *L. carinii* mf to a concentration greater than 10 mM Ca^{2+} , endopeptidase (5.8 units/ml), or papaya extract protease (3.0 units/ml) induced 90-100 % exsheathment [172]. In the experiments described here, mf were exposed to 1 mg/ml Pronase, which stimulated 98% exsheathment after 10 minutes incubation (Figure 3.1). The exsheathment of the mf by exposure to protease is presumably caused by the partial digestion of the

sheath. It is important to note mf exsheathed by exposure to Ca^{2+} or endopeptidase inoculated into susceptible female mosquitoes are viable and continue to develop normally [65].

The results of the *in vitro* culture experiments showed behavioural differences between the two groups of mf following culture. This observation suggested that the mf may respond to the different culture conditions and that perhaps the behavioural differences would be reflected with differences in gene expression between the two populations. Proudfoot *et al* (1993) [74], showed that changes in the surface properties of *B. pahangi* L3 occurred after less than ten minutes culture in mammalian medium (RPMI), versus insect medium (Grace's) [73]. These changes were too rapid to be the initiation of an impending moult, and were thought to be associated with an induced change in the parasite caused by external stimuli. Interestingly the behavioural changes between mf cultured in mammalian and vector conditions reported here, were similar to those reported by Proudfoot *et al*.

The RT-PCR strategy used to produce a cDNA library has advantages if the amount of starting material is limiting. This is particularly relevant when working with parasites such as *Brugia spp* where no culture system is available that allows the development of the worm and where a relatively small amount of parasite material is available from infected animals. The "stockpiling" of parasites from a life cycle stage of interest for subsequent analysis is one way of overcoming this problem, but, in situations where time is a factor to be considered, the construction of a cDNA library as described can be a relatively quick and simple method for the isolation of genes of interest.

The problems associated with the use of an SL1 RT-PCR library are: on average inserts ligated into the vector are of smaller size compared to a conventionally made library, and the relative abundance of mRNA transcripts *in vivo* and the corresponding cDNAs in the library may not be truly comparable. The average insert size of the *B. pahangi* mammalian mf library constructed in this project was approximately 500 bp. The insert size was smaller than anticipated, presumably because some of the cDNAs are truncated and not full-length transcripts, a limitation inherent in the PCR technique. Blaxter *et al* (1996), by comparison of two *B. malayi* L3 libraries constructed using either conventional, or SL1-oligo (dT) RT-PCR methods, showed that the PCR library contained inserts which were an average of 30% smaller than the conventional library, 700 bp compared to 1Kb in PCR and conventional libraries, respectively [129]. Another obvious limitation of the technique is that the SL1 PCR selects for those genes that are SL1 *trans*-spliced during mRNA maturation. In this project the advantages of ease of construction from small amounts of material was considered to outweigh the disadvantages. The presence of a spliced leader (SL) sequence at the 5' end of mRNAs was first described in the kinetoplastid protozoan *Trypanosoma brucei* [177]. In Trypanosomes all mRNAs possess a 39 nucleotide SL known as the mini-exon sequence. This absolute requirement for the SL sequence in the Trypanosomes is due to the polycistronic organisation of transcripts that are processed to monocistronic units by *trans*-splicing with SL and polyadenylation. The only metazoans to exhibit *trans*-splicing of SL sequences are the platyhelminths and nematodes. *Trans*-splicing has been found to be universal throughout all nematodes studied to date [178]. SL sequences were first discovered in the nematodes on actin mRNAs of *C. elegans*

[179]. The abundance of this SL sequence termed SL1 on *C. elegans* mRNAs has recently been predicted to be >70%. [110] while 80-90% of *Ascaris* mRNAs are predicted to contain SL1 [111]. The SL1 sequence of *Brugia* was initially shown to be present on the mRNA for a 63 kDa antigen and was found to be identical to the 22 nucleotide SL1 sequence as described in *C. elegans* [180].

The two-step PCR protocol was designed to overcome some of the problems mentioned above. By amplifying through 10 cycles, size fractionating, and then using additional rounds of PCR it was hypothesised that early preferential amplification of some transcripts at the expense of others, leading to a swamping of the reaction mixture with a few highly amplified cDNAs, would be avoided. In this way it was hoped that the relative abundance of cDNA species *in vitro* to mRNA species *in vivo* would remain at comparable levels and that this method would isolate truly differentially expressed genes. Although this was the aim it appears that, as the insert size ranged from 200-900bp and the size separation columns should have removed cDNAs of < 400bp, the size separation step was less than optimal.

Prior to carrying out the differential screen the library was subjected to various characterisation procedures. Using the mechanical replicator, 940 plaques were plated but no differentially hybridising clones were isolated. This demonstrated that in excess of 940 plaques would need to be screened in order to isolate differentially expressed genes. The total number of differentially hybridising cDNAs isolated in this study was 21 from a screen of 2×10^4 pfu, ($\leq 0.1\%$). This number agrees with the prediction of the initial experiment that the number of differentially hybridising cDNAs in the library is approximately 1:1000.

One of the major differences in the culture systems used to mimic the mammalian and vector hosts, was temperature, 37°C versus 28°C respectively. As such it was a concern that heat shock proteins might be over-represented in the library. However a plaque lift of the 81 primary screen positives gridded by the mechanical replicator was hybridised with an *hsp90* cDNA and only one positive was observed (Figure 3.8). As the *hsp90* mRNA has previously been shown to be up-regulated in the mammalian-derived mf, this increased confidence in the culture systems. Furthermore when the 81 positives were screened against a small heat shock protein cDNA, no hybridisation was observed (data not shown), suggesting that heat shock proteins were not over-represented in the library.

The use of the reverse northern method had advantages over a tertiary plaque screen as it was possible to use equal amounts of cDNA, thus the screen was more quantitative. These factors together gave confidence that the culture system did influence gene expression in a manner that mimicked *in vivo* events, and that the initial aim of isolating cDNAs, representing mf genes which are differentially expressed in mammalian and mosquito hosts was possible by this technique.

From the screen the majority (9/21) of differentially expressed genes are those encoding ribosomal proteins. The presence of high numbers of ribosomal protein genes in the library may be due to the method of library construction. Blaxter *et al* (1996) compared the percentage of ribosomal protein cDNAs in conventional and SL1 RT-PCR libraries of *B. malayi* L3 [129]. In the RT-PCR library 24 % of the total cDNAs represented ribosomal protein genes compared to 10 % in the conventional library. This trend has also been seen in other EST projects; for example in *Trypanosoma brucei*, an SL1 PCR library contained 19% ribosomal protein cDNAs

[129]. The prevalence of these cDNAs in the PCR libraries is likely to be due to a combination of two factors. Firstly the high concentration of ribosomal protein mRNAs in the original population; 10% of all cDNAs from *B. malayi* libraries prepared from non-L3 life-cycle stages represent ribosomal proteins. Blaxter speculated that the high abundance of ribosomal proteins seen in certain life-cycle stages may represent a store for future high levels of protein synthesis [129]. This explanation may also apply to the mf. A second consideration is the relatively small size of ribosomal protein mRNAs. The largest ribosomal protein mRNA (ribosomal protein S10) from the *B. malayi* L3 library is 580 bp, and the average size of the 43 described to date is 381bp. It has been shown that the size of the target sequence can dramatically effect the yield of product by PCR. By studying gain versus allele length curves the molar yield of a 1Kb allele will be 18 times higher than that of a 6kb allele after 10 cycles, increasing to a 1300 fold excess after 25 cycles [181]. These figures could explain why the PCR libraries have a higher percentage of small size fragments including the ribosomal proteins.

It is generally assumed that ribosomal proteins would be constitutively expressed, however a number of reports do show that specific ribosomal proteins are differentially expressed during development [182-184]. This raises the interesting possibility that different species of ribosomal protein may be utilised at different stages of development, although this will not be investigated further in the present study. The fact that abundant cDNAs were being isolated may mean that less abundant differentially expressed transcripts are being missed. This was a concern in the present investigation, but as a number of potentially interesting cDNAs, were

isolated it was decided to proceed with these. The following chapters aim to further characterise the cDNAs, *mmc-1* and *vmc-2*.

CHAPTER FOUR

4.1 Introduction.

In the previous chapter the cloning of a number of differentially expressed genes was described. This chapter describes further studies of the gene *vmc-2*, one of the cDNAs that appeared to be up-regulated in the mf exposed to mosquito-like conditions *in vitro*.

Although the morphological changes undergone by the mf in the mosquito host have been described in detail almost nothing is known of the molecular mechanisms which underpin these changes. During the first 48 hours in the mosquito the mf undergo an extraordinary metamorphosis. Within 1 hour of feeding the mf are present in the flight muscles of a susceptible host, the mf have lost their sheath and lie in the muscle fibres and become sluggish. By 48 hours p.i the mf have begun to shorten in length and increase in width (190 μm X 8 μm at 24 hours) and by day three p.i. the mf is at its minimum length (172 μm X 16 μm) [9], as described in 1.1.2. The analysis of genes such as *vmc-2* may lead to greater understanding of the development of the parasite from mf to infective L3. Likewise due to the nature of the intracellular infection very little information is available about the biochemistry and physiology of the parasite in the mosquito vector [185, 186]. The characterisation of genes such as *vmc-2* might therefore provide some insight into the biology of *B. pahangi* mf in the mosquito host.

4.2 Results

4.2.1 Nucleotide sequence information.

Three independent *vmc-2* excised clones (see section 2.5.3) were sequenced to determine an accurate consensus sequence for the *vmc-2* gene (section 2.2.20). The

sequence of *vmc-2* is shown in Figure 4.1a. The *vmc-2* library clone contains the conserved nematode SL-1 sequence shown in bold. The string of adenosine residues at the 3' end of the gene is unlikely to be the true Poly-A tail as the poly-adenylation signal sequence (AATAAA) is not present upstream. Translation of the *mmc-1* sequence using the GCG genomics program (Genetics Computer Group Madison, Wisconsin) revealed a single extended reading frame, but this does not possess a stop signal (Figure 4.1b). Therefore *vmc-2* is likely to be a truncated sequence caused by the internal priming of oligo (dT) in the PCR reactions. Northern blot analysis described in section 4.2.4 confirms this assumption, while attempts to isolate the full length transcript of *vmc-2* are described in 4.2.5

The nucleotide sequence of *vmc-2* was compared to the non-redundant databases (Genbank, EMBL, DDBJ and PDB) at the NCBI website (<http://www.ncbi.nlm.nih.gov/blast/>) using the BLAST algorithm [124]. The only significant matches were restricted to the SL-1 splice leader sequence and when this portion was removed no significant matches were returned. The predicted amino acid sequence (Figure 4.1b) was used to search the GenBank and SwissProt data bases for homologous peptide sequences. The most significant match was to a *C. elegans* hypothetical 90.8 kDa protein TO5H10.7 localised to chromosome II (GenBank Q10003), which showed 44.8 % identity over the 79 amino acid peptide. The region of similarity was restricted to a glycerophosphoryl diester phosphodiesterase (GLP) domain. The GLP gene products are involved in glycerol metabolism in bacteria [187], have been reported to be important antigens for diagnosing *Borrelia hermsii* relapsing fever [188] and are protective antigens in infectious syphilis [189]. The TO5H10.7 sequence was retrieved from the *C. elegans* genome database at the

Figure 4.1a Nucleotide sequence of *vmc-2*.

```
1      GGTTTAATTACCCAAGTTTGAGGTCAAGCACTTACTGCCCAAATGTTCAGT
51     TGCACCACGCACAGTGCTTCATTTTTTGGTGGAAGTTGAAGCAGTACGAC
101    CGTGGGAGTATGTATTTGTTACAGGTTTCATTGCCAGTTTTAGGTGGTTGG
151    TTACCATCTAATGCGTTCGTTTTATTTCCGGATCCGGACTCTGCTAGGCA
201    ACGTTGGGAAGGTGAAGTAGAAATTGGATTGGATCCAGTAAAATTCCGTT
251    ATTTCATTGGATACTATTTGCACAGTGGTAAAAAAAAAAAAAAAAAAAAA
```

Figure 4.1a

The consensus sequence was derived from multiple clones from the mammalian mf library. These were isolated and sequenced in both directions using T7 and M13 reverse sequencing primers. The conserved SL-1 sequence is shown in bold at the 5' end of the gene. The putative translational start codon at position 45 is double underlined, there is no stop codon within the sequence of the proposed ORF.

Figure 4.1b Predicted amino acid sequence of *vmc-2* ORF.

```
1      MSVAPRTVLHFLVEVEAVRPWEYVFVTGSLPVLGGWLPSNAFVLFPDPDS
51     ARQRWEGEVEIGLDPVKFRYFIGYYLHSG
```

Figure 4.1b

The proposed amino acid sequence, generated by translation of the nucleotide sequence, from ATG at position 45. The predicted protein consists of 79 amino acids, from the start codon to the end of the isolated sequence.

Sanger centre and analysed for domain profiles using the Pfam algorithm [190] (<http://www.sanger.ac.uk/Software/Pfam/search.shtml>). The Pfam-B domain 35977 was found to be homologous. The genes of known function that contain this domain were bacterial GLP genes. Figure 4.2 shows a ClustalW alignment of the known GLP gene products from various bacterial species. As can be seen the sequences show a high degree of similarity along the length of the peptides. When the *vmc-2* peptide sequence was added to the alignment it did not align well with the other GLPs (Figure 4.3). The *vmc-2* ORF needs especially large extended gaps to allow any homology and therefore this sequence is not believed to be a true GLP.

The T05H10.7 sequence is a hypothetical predicted protein although a number of cDNAs have been sequenced that correspond to it. One named yk24e8.5 corresponds to the region of T05H10.7 showing 48 % identity to *vmc-2* at the amino acid level and may therefore represent the *C. elegans* homologue of *vmc-2*. No homology is seen at the nucleotide level which may represent the divergence of the sequences or be due to differences in codon bias between the two species. Comparison of *vmc-2* by the Wu-Blastn algorithm to the parasite genome server at the EBI (<http://www2.ebi.ac.uk/cgi-bin/parasiteblast2>) produced no significant matches to other nematodes or to *B. malayi* sequences. As the predominant source of data in the filarial database are ESTs, which, at least initially, represent abundant cDNAs, this may suggest that *vmc-2* is not an abundant mRNA or is poorly represented in the libraries sequenced.

Figure 4.2
ClustalW alignment (MacVector, Oxford Molecular) of five bacterial GLPs. Matched residues are boxed, identical matches are shown in bold.

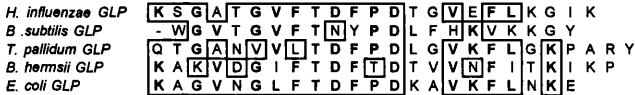


Figure 4.3 Multiple sequence alignment of *vmc-2* ORF and bacterial GLPs.

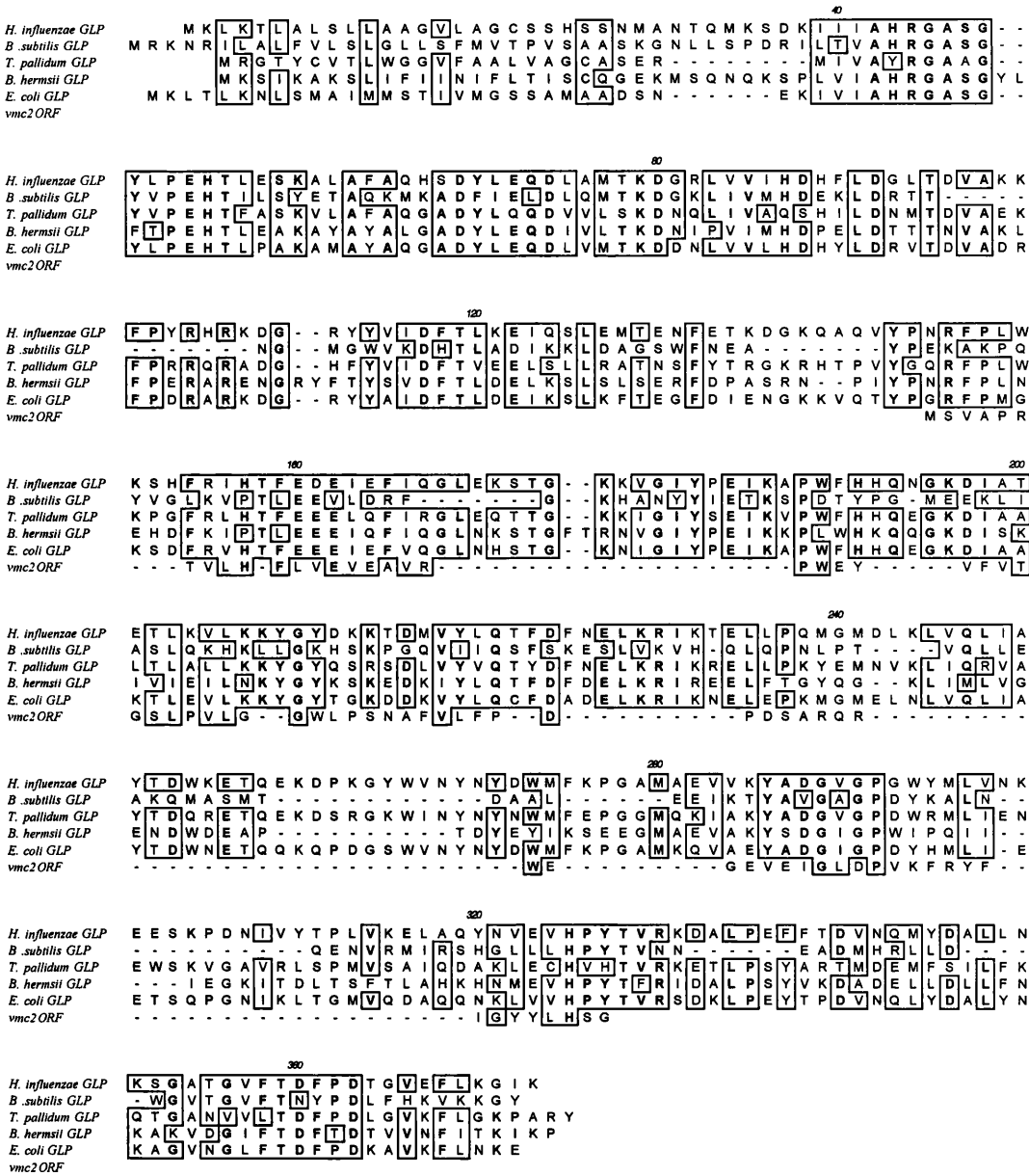


Figure 4.3 ClustalW alignment (MacVector, Oxford Molecular) of *vmc-2* ORF and bacterial GLP peptide sequences. Matched residues are boxed, identical matches are shown in bold.

4.2.2 Generation of gene specific probes.

A *vmc-2* cDNA fragment (*vmc-2F1-R1*) was generated by PCR using two gene specific primers, *vmc-2F1* and *vmc-2R1* designed from the sequence shown in Figure 4.1 (for primer sequences see Table 2.1). cDNA was amplified by a standard PCR protocol using mammalian-derived mf first strand cDNA as a template. A single cDNA product of 252bp named *vmc-2F1-R1* was obtained, see Figure 4.4. The purified DNA was radiolabelled with $^{32}\text{P}\alpha\text{-dCTP}$ and was confirmed to be *vmc-2* by Southern hybridisation to a *vmc-2* containing plasmid. A PCR using the same primers was conducted using 200 ng of *B. pahangi* genomic DNA as a template for amplification. A band of approximately 1 kbp (Figure 4.5) was produced that hybridised to the *vmc-2* cDNA probe. This band named *vmc-2F1-R1*gen was cloned into a TA vector (Invitrogen) and stored at -20°C until later use.

4.2.3 Comparison of *vmc-2* cDNA and genomic PCR fragments.

Three independent PCR clones of the *vmc-2* genomic PCR fragment, isolated as described above, were sequenced on three occasions on both strands using a Li-COR sequencer. The consensus sequence compiled from the sequence information is shown as Figure 4.6. By comparison of the sequences produced by cDNA and genomic DNA PCR a map of the predicted intron and exons of the *vmc-2* genomic fragment was produced. A single intron of 716 bp is present in the PCR fragment from positions 167-883 and is shown in bold.

Figure 4.4a Positions of *vmc*-2F1 and *vmc*-2R1 primers.



Figure 4.4b Generation of *vmc*-2F1-R1 gene specific cDNA probe.

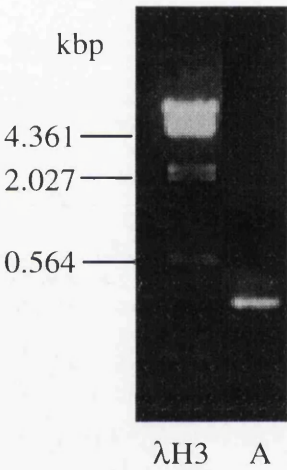


Figure 4.4b

1µl of mammalian-derived mf first strand cDNA was PCR amplified using *vmc*-2F1 and *vmc*-2R1 primers as follows: thirty cycles of: 94°C 1 minute, 60°C 1 minute, 72°C 1 minute. Amplified products were size separated on a 1% agarose gel. A single band of predicted size, 252bp was observed, this was excised and purified using Qiagen gel purification kit. When tested it specifically hybridised to a plasmid containing *vmc*-2.

λH3 = *Hind* III digested λ DNA molecular size markers.

A= *vmc*-2F1-R1 PCR.

Figure 4.5 *vmc-2F1-vmc-2R1* genomic PCR.

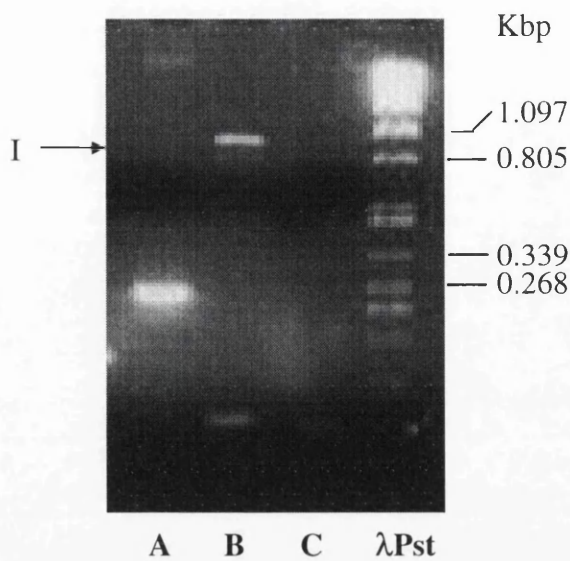


Figure 4.5

200 ng of *B. pahangi* genomic DNA was amplified by PCR using the *vmc-2F1* and *vmc-2R1* primers as follows: Thirty cycles of: 94°C 1 minute, 60°C 1 minute, 72°C 3 minutes.

The amplified products were size separated in a 2 % agarose gel. A band of approximately 1 kb, marked I was isolated and purified.

A = Positive control *vmc-2* plasmid PCR *vmc-2F1* and *vmc-2R1* primers.

B = 200 ng Genomic DNA PCR *vmc-2F1* and *vmc-2R1* primers.

C = No template negative control.

λPst = *Pst* I digested λ DNA molecular size markers.

Figure 4.6. Sequence of *vmc-2F1-R1* genomic PCR fragment.

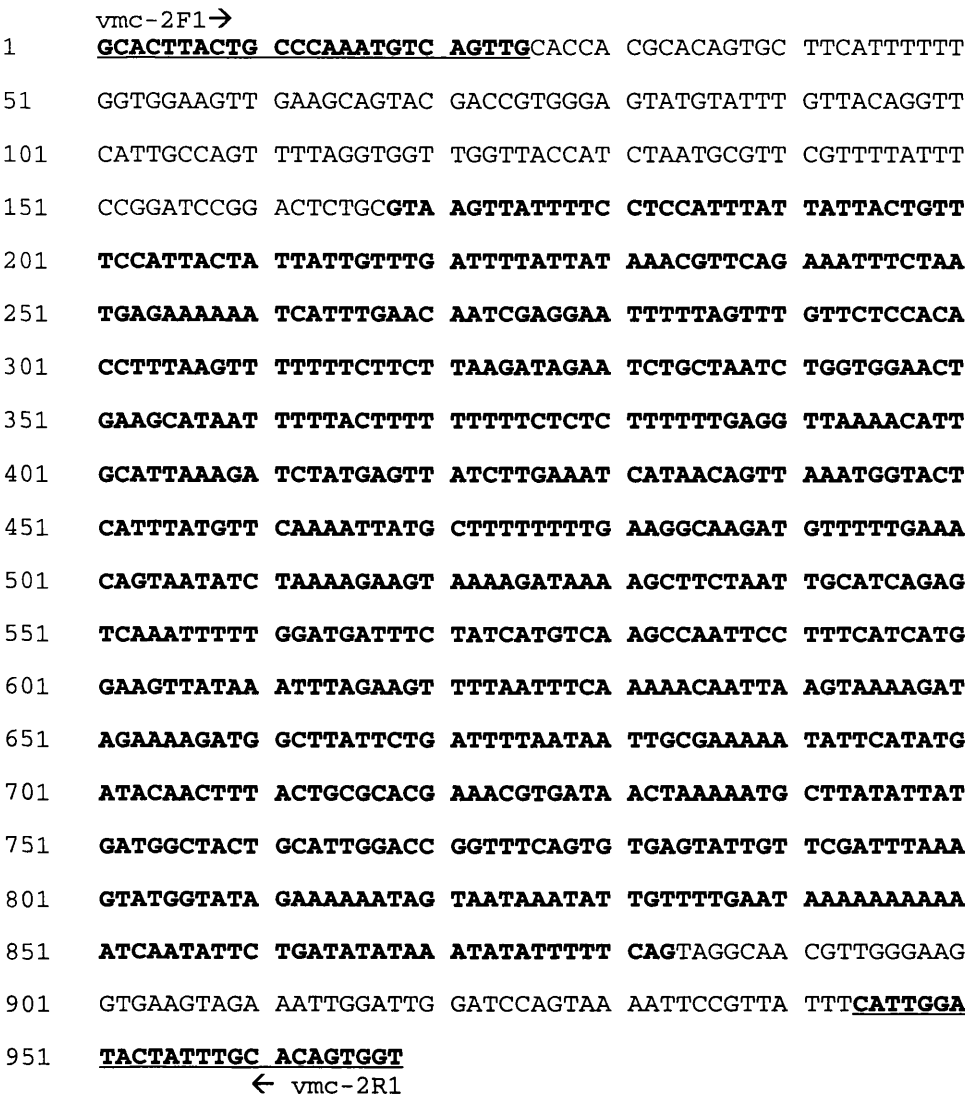


Figure 4.6

The genomic *vmc-2F1-R1* PCR product (Figure 4.5) was ligated into a TA vector (Invitrogen) and sequenced on both strands on three occasions. The consensus sequence is given above. The positions of the two primers are shown in bold and are underlined. By comparison of the cDNA and genomic sequences a single intron was detected, shown in bold.

4.2.4 Northern blot analysis of *vmc-2*.

2×10^6 mature mf collected from the peritoneal cavity of an infected jird were purified from contaminating host cells as described in section 2.1.4. The mf were then cultured in one of two ways, 2 hours at 37°C in MEM, (mammalian-derived mf) or 2 hours at 28°C in Grace's insect culture medium (vector-derived mf). Three µg of RNA extracted from the mf using standard methods (2.2.15) was separated on a 1.5 % agarose gel. Comparable quantities of RNA were used from each sample, as determined by ethidium bromide staining of the RNA and visualisation under UV light (Figure 4.7a). The RNA was transferred to nylon membrane and then probed with the *vmc-2*F1-R1 cDNA gene-specific probe, and washed to high stringency at 65°C. The radiolabelled blot was then exposed to autoradiographic film for 14 days after which time it was possible to see a faint signal.

The ethidium bromide stained gel (Figure 4.7a) shows approximately equal loading of RNA in each lane, whilst Figure 4.7b shows the autoradiograph. The pattern of expression follows that seen in the differential screen from which the *vmc-2* fragment was isolated, i.e. a greater abundance in vector-derived mf mRNA compared to that of mammalian-derived mRNA, although the degree of upregulation is very slight. The relatively weak signal obtained presumably reflects the low abundance of *vmc-2* mRNA in the total RNA population of the mf. The approximate size of the transcript is 600 bp (marked I), confirming that the cDNA obtained from the mf library is a truncated fragment and that approximately 350 bp of sequence is missing. The very weak band in the mammalian-derived RNA appears slightly smaller than the band in the vector-derived mf. This may be due to a difference in the size of transcript under the different conditions or may be due to membrane slippage

Figure 4.7a.

RNA was extracted using standard techniques from 2×10^6 mf cultured for 2 hours in either Grace's culture medium at 28°C (vector-derived mf) or MEM at 37°C (mammalian-derived mf). 3µg of each sample was separated in a 1.5% agarose gel and stained with ethidium bromide.

M = RNA markers (Gibco)

A = *in vitro* cultured mammalian-derived mf RNA.

B = *in vitro* cultured vector-derived mf RNA.

Figure 4.7b.

RNA samples shown in Figure 4.7a were transferred to a nylon membrane and hybridised with the *vmc*-2F1-R1 cDNA probe. The blot was washed to high stringency at 65°C as follows: 2 x 10 minutes 2 x SSC 0.1 SDS, 2 x 10 minutes 1 x SSC 0.1 SDS, 2 x 10 minutes 0.1 x SSC 0.1 SDS. The blot was then exposed to autoradiographic film for 14 days at -70°C. The approximate size of the *vmc*-2 transcript is 600bp, marked I.

Figure 4.7a Ethidium bromide stained RNA.

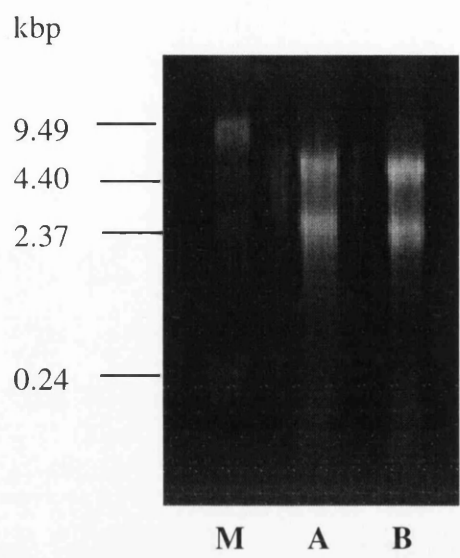
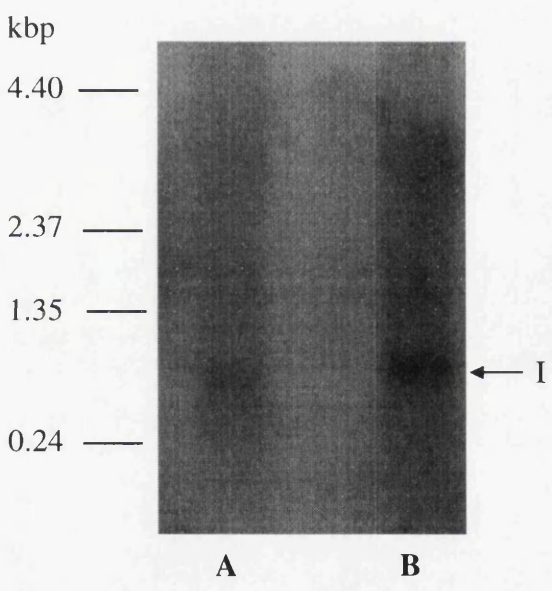


Figure 4.7b *vmc-2* northern blot.



4.2.5 Attempts to obtain a full length transcript of *vmc-2*.

The first attempts to clone the full length transcript of *vmc-2* used a PCR of higher stringency on first stand cDNA which had been produced using a lower concentration of adapted oligo (dT) primer. It was hoped that by decreasing the concentration of the primer from 0.5 μ M to 0.2 μ M, the proportion of primer that annealed non-specifically would decrease. In order to further increase the specificity of the PCR step a higher annealing temperature of 62°C was also used.

A hot start PCR amplification protocol using *vmc-2*F1 and APA primers was conducted under standard conditions (section 2.2.2). Unfortunately this did not produce a longer length product and so an alternative approach was adopted. PCR was conducted on a variety of different cDNA libraries available in the laboratory; these included two PCR libraries constructed from post-infective L3 stage parasites [79, 80], and a conventionally constructed adult stage library [191]. PCR was conducted using the *vmc-2*F1 gene specific primer and a vector specific primer, T7. In each of these experiments a positive control plasmid containing the *vmc-2* fragment was also amplified to allow the detection of any additional sequence produced. Unfortunately this approach was also unsuccessful producing products of the same size as the control reaction (Figure 4.8). The faint lower band (marked I) is thought to be due to the formation of “primer-dimers” between the primer pairs.

A similar approach was then taken using a *B. pahangi* genomic library in EMBL3 (provided by J. Hirzmann, University Giessen, Giessen, Germany) as the template for PCR. *vmc-2*F1 and *vmc-2*R1 primers were used in combination with EMBL forward and reverse vector primers. It was hoped that the 3' end of the genomic clone could be isolated and subsequently sequenced. The PCR products

Figure 4.8a

This experiment represents an attempt at cloning the 3' end of the *vmc-2* transcript by PCR on two post infective L3 stage libraries using a combination of gene specific and vector primers. 5 µl of each library was amplified as follows: thirty cycles of: 94°C 1 minute, 58°C 1 minute, 72°C 3 minutes. The amplified products were size separated in a 1% agarose gel. The very slight increase in the size of band in lanes A and B is due to one primer being situated on the vector outwith the cloned insert.

λ*Pst* = *Pst* I digested λ DNA molecular size markers.

A = 24 hour p.i L3 library. *vmc-2F1*, T7 vector primers.

B = 3 day p.i L3 library. *vmc-2F1*, T7 vector primers.

C = *vmc-2* plasmid 1:100 dilution positive control. *vmc-2F1*, *vmc-2R1* primers.

D = No DNA template negative control. *vmc-2F1*, *vmc-2R1* primers.

Figure 4.8b

PCR under the same conditions described above using an adult library.

λH3 = *Hind* III digested λ DNA molecular size markers.

A & B = 5 µl Adult library template *vmc-2F1*, T7 vector primers.

C = *vmc-2* plasmid 1:100 dilution positive control. *vmc-2F1*, *vmc-2R1* primers.

D = No DNA template negative control. *vmc-2F1*, *vmc-2R1* primers.

Figure 4.8a PCR of *B. pahangi* post-infective L3 cDNA PCR libraries to isolate 3' end of *vmc-2*.

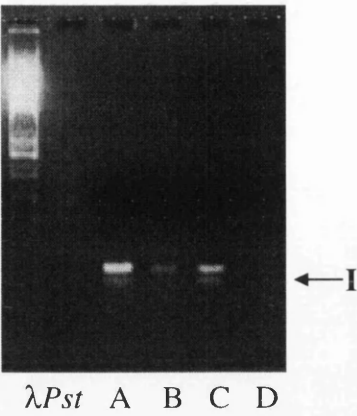
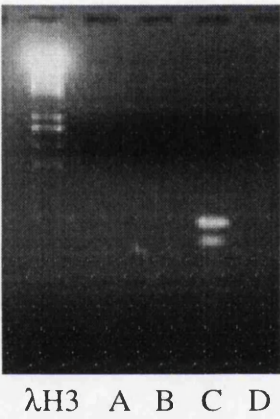


Figure 4.8b PCR of *B. pahangi* adult stage library to isolate the 3' end of *vmc-2*.



were blotted onto nylon membrane and probed at 60°C with the *vmc-2F1-R1* gene specific probe. Figure 4.9 shows the results of this experiment. Lanes A to D show various combinations of primers, none of which yielded any product. Lane E is a control PCR using *vmc-2F1* and *vmc-2R1* primers which produced a band of predicted size, 968 bp (marked i). Lanes F and G are control reactions using genomic DNA as the template. The additional bands seen in lanes E and G are possibly due to incomplete extension of amplified products leading to a series of truncated fragments to which part of the probe can hybridise to, leading to the results seen. This seems to be related to the concentration of template DNA used, compare lane F (20 ng DNA) and lane G (100 ng DNA). The bands marked ii are thought to be “primer-dimer” artefacts formed during the PCR caused by complementarity between the primers.

In a final attempt to isolate the 3' end of *vmc-2* a Marathon cDNA amplification kit (Clontech Palo Alto CA USA) was utilised. The Marathon kit, as in previous attempts, relies on the use of an adapter primer that will amplify from the true Poly-A tail at the 3' end of the mRNA. An overview of the protocol is given in Figure 4.10. Total RNA was isolated from two batches of approximately 100 adult female worms. Approximately 4 µg of total RNA was used to synthesise the first strand cDNA using the modified oligo (dT) primer provided. This was followed immediately by the second strand cDNA synthesis using T4 DNA polymerase. The blunt ended double stranded cDNA products were then ligated to the Marathon cDNA adapter provided in the kit for 3.5 hours at 21°C. A control PCR was conducted on the cDNA template provided with the kit using combinations of an adapter primer, AP1, that corresponds to the ligated cDNA adapter and two control primers. Figure 4.11 shows the results of this experiment. Neither of the control

Figure 4.9 PCR of *B. pahangi* lambda EMBL3 genomic DNA library to obtain additional 3' sequence information.

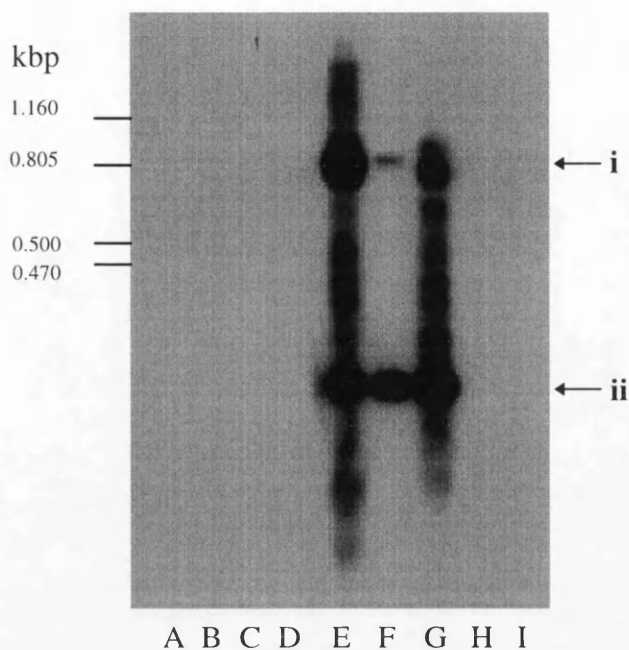


Figure 4.9

5µl of amplified library was amplified by PCR under the following conditions: thirty-five cycles of: 94°C 1 minute, 55°C 1 minute, 72°C 3 minutes. Amplified products were size separated in a 2 % agarose gel, and then transferred to nylon membrane and probed with *vmc-2* gene specific cDNA probe. Washed at 60°C as follows: 2 x 10 minutes 2 x SSC 0.1% SDS, 1x 10 minutes 1 x SSC 0.1% SDS, 1 x 10 minutes 0.1 x SSC 0.1%SDS. The autoradiograph was exposed for 24 hours.

i = Predicted 968 bp fragment produced by *vmc-2*F1-R1 PCR.

ii = “Primer-dimers” formed by the annealing of the primer pairs.

A = 5 µl amplified genomic library PCR *vmc-2*F1, EMBL forward primers.

B = 5 µl amplified genomic library PCR *vmc-2*R1, EMBL forward primers.

C = 5 µl amplified genomic library PCR *vmc-2*F1, EMBL reverse primers.

D = 5 µl amplified genomic library PCR *vmc-2*R1, EMBL reverse primers.

E = 5 µl amplified genomic library PCR *vmc-2*F1, *vmc-2*R1 primers.

F = 20 ng genomic DNA PCR *vmc-2*F1, *vmc-2*R1 primers.

G = 100 ng genomic DNA PCR *vmc-2*F1, *vmc-2*R1 primers.

H = 5 µl amplified genomic library PCR EMBL forward, EMBL reverse primers.

I = *vmc-2*F1, *vmc-2*R1 No template DNA negative control.

Figure 4.10a Flow diagram of Marathon RACE protocol.

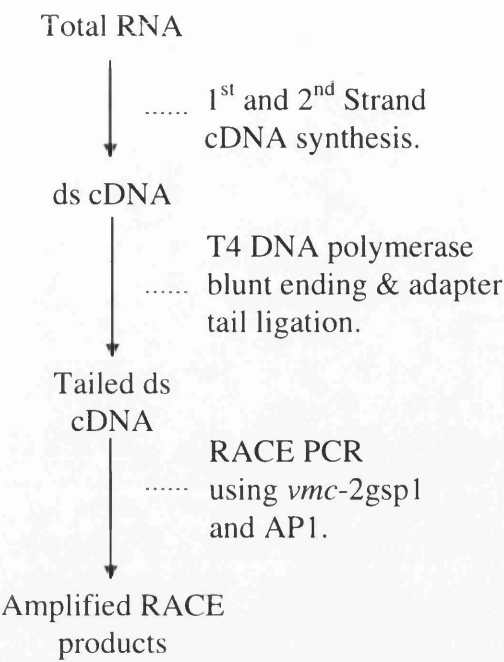


Figure 4.10a
A simplified flow diagram of the Marathon RACE protocol.

Figure 4.10b Schematic of primer positions used in Marathon RACE.



Figure 4.10b
A schematic diagram showing the positions of primers used in the RACE techniques.
Sizes of cDNAs produced by PCR using primer pairs.
vmc-2F1 - *vmc-2R1* = 252 nt.
vmc-2gsp1 - *vmc-2R1* = 199 nt.

Figure 4.11 Marathon RACE kit control PCR.

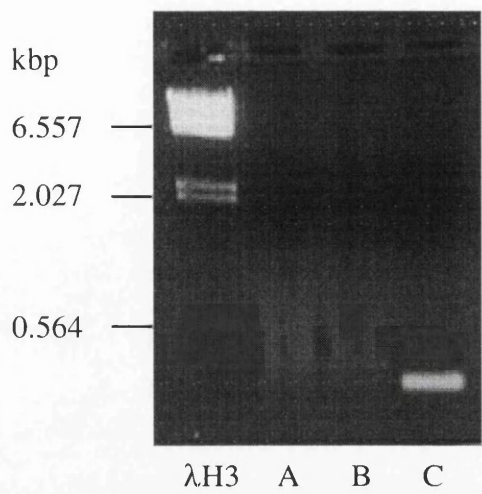


Figure 4.11

A control PCR was conducted as described in the Clontech Marathon PCR manual. Touch-down PCR conducted as follows: Five cycles of: 94°C 30 seconds, 72°C 4 minutes; five cycles of: 94°C 30 seconds, 70°C 4 minutes; twenty five cycles of: 94°C 20 seconds, 68°C 4 minutes. 10µl of the 50µl reaction was separated on a 1 % agarose gel.

- λH3 = *Hind* III digested λ DNA molecular size markers.
- A = 5' TFR primer, API primer 5µl control cDNA template. Predicted size 2.6kb.
- B = 3' TFR primer, API primer 5µl control cDNA template. Predicted size 2.9kb.
- C = 5' TFR primer, 3' TFR primer 5µl control cDNA template. Predicted size 0.3kb.

reactions (lanes A and B) using AP1 produced products of the predicted size (2.6 and 2.9kb), but the two internally placed primers did produce a band of correct size (0.3kb, lane C) that could be seen on an ethidium bromide stained gel. Therefore it was assumed that the ligation of the adapters or the PCR was not optimal. As amounts of the Marathon kit were limiting the test reactions were conducted. The cDNA products were amplified using the adapter primer, and two *vmc-2* gene specific primers, *vmc-2F1* and *vmc-2gsp1*, each at 0.2 μ M final concentration. The results of this experiment are shown in Figure 4.12a No clear bands were seen in the experimental lanes, but the control lanes using *vmc-2F1/R1* primers produced bands of predicted size. The amplified products were transferred to nylon membrane and probed with the *vmc-2F1-R1* cDNA probe. The results are shown in Figure 4.12b. In each of the experimental lanes a band of slightly greater size than *vmc-2F1-R1* can be seen. An aliquot of each of these was re-amplified using *vmc-2gsp1* and AP1 primer pairs. A control using *vmc-2F1* and oligo (dT) primer primers was also used. The results of the PCR are shown in Figure 4.13. Both PCRs show a predominant band of approximately 250 bp (Marked I) and also a faint band above the 564 bp marker (Marked II). It was assumed that the band of 250 bp was due to the modified oligo (dT) primer used in first strand synthesis hybridising to an adenosine rich region in the gene, as had happened in the original *vmc-2* cDNA fragment. The larger size band was therefore assumed to be amplification from the true Poly A tail that a smaller proportion of the modified oligo (dT) had annealed to in both first strand synthesis procedures. The smaller size of the band produced by *vmc-2gsp1*-AP1, (lane B) compared to *vmc-2F1*-oligo (dT), (lane A) is due to the internal position of *vmc-2gsp1* relative to *vmc-2F1* (Figure 4.10). The two larger bands (marked II) were

Figure 4.12a Marathon RACE PCR.

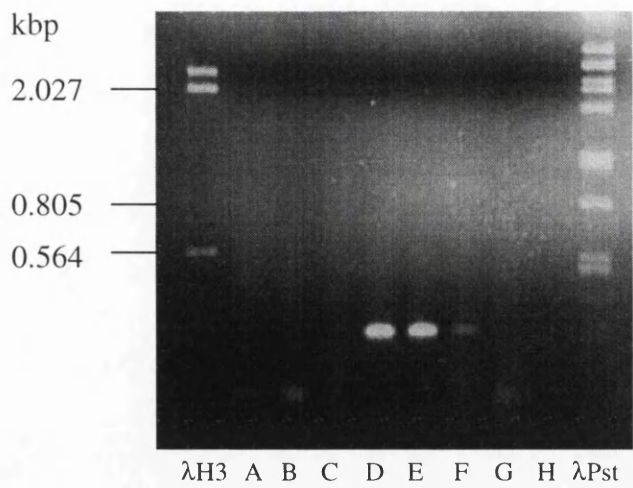


Figure 4.12b Marathon RACE test PCR Southern.

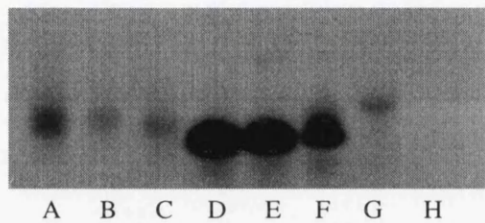


Figure 4.12a

Ethidium bromide stained agarose gel showing cDNA products amplified by Marathon PCR. "Touch-down" PCR conducted as follows: Five cycles of 94°C 30 seconds, 68°C 4 minutes; five cycles of 94°C 30 seconds, 66°C 4 minutes; twenty five cycles of 94°C 20 seconds, 64°C 4 minutes. 5µl of the 50µl reaction was run on a 1.2 % agarose gel.

λH3 = *Hind* III digested λ DNA molecular size markers.

λPst = *Pst* I digested λ DNA molecular size markers.

A = *vmc*-2gsp1, AP1 1:50 cDNA dilution.

B = *vmc*-2gsp1, AP1 1:250 cDNA dilution.

C = *vmc*-2gsp1, AP1 1:1 cDNA dilution.

D = *vmc*-2F1, *vmc*-2R1 1:50 cDNA dilution.

E = *vmc*-2F1, *vmc*-2R1 1:250 cDNA dilution.

F = *vmc*-2F1, *vmc*-2R1 1:1 cDNA dilution.

G = *vmc*-2F1, AP1 1:50 cDNA dilution.

H = *vmc*-2gsp1, AP1 No template DNA control.

Figure 4.12b

The gel shown in Figure 4.12a was transferred to nylon membrane and probed with the *vmc*-2F1-R1 gene specific cDNA probe. The resulting blot was washed to high stringency at 65°C. Lanes A-H as labeled above.

Figure 4.13 Oligo (dT) 3' Race PCR.

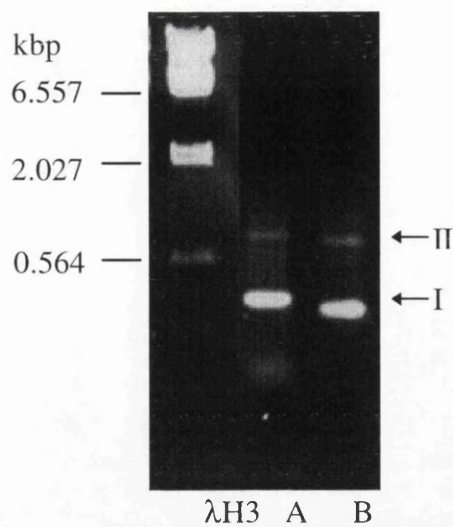


Figure 4.13
PCR of first strand cDNA using *vmc-2gsp1*, oligo (dT) and *vmc-2F1*, oligo (dT).
PCR under the following conditions: Thirty five cycles of: 94°C 1 minute, 62°C 1 minute, 72°C 3 minutes. 10 µl of the 50 µl reaction was run on a 2 % agarose gel. The major bands produced by the PCR are marked I and II.

λH3 = *Hind* III digested λ DNA molecular size markers.
A = *vmc-2F1*, oligo d(T) PCR.
B = *vmc-2gsp1*, oligo d(T) PCR.

excised from the gel ligated into TA vector, and sequenced using M13 reverse sequencing primer. Unfortunately the bands cloned were unrelated sequences and not 3' extensions of the previously cloned *vmc-2*.

4.2.6 Southern blot analysis of high molecular weight DNA.

To determine whether *vmc-2* is a single copy gene, or if it is part of a larger gene family, Southern blot analysis of high molecular weight DNA was conducted. Genomic DNA was isolated from approximately 300 mixed sex adult *B. pahangi*. 20 µg of DNA was digested at 37°C overnight in a 1 X concentration of the appropriate Restriction buffer using 10 units of restriction enzyme (Gibco Life Technologies). Three enzymes were selected for analysis of *vmc-2*, *EcoR* I, *Hind* III and *Bam*H I. There is no *EcoR* I restriction site in the *vmc-2*F1-R1 genomic probe, one *Hind* III site at position 434 and two *Bam*H I sites at positions 44 and 811 bp (Figure 4.14). If *vmc-2* is a single copy gene then a single band of hybridisation should be seen in the *Eco* RI digested lane, two bands in the *Hind* III lane and three bands in the *Bam* HI lane. The Southern blot was hybridised at 50°C with 25 ng of *vmc-2*F1-R1 genomic fragment radiolabelled with ³²P. Following hybridisation the blot was washed for 4 x 10 minutes in 2 x SSC, 0.1% SDS, and exposed to X-ray film at -70°C. Figure 4.15 shows the ethidium bromide stained DNA smear and the resulting autoradiograph. A single band of approximately 6.5 kbp was seen in the *EcoR* I lane, and two bands were seen in the *Bam*H I (approximately 0.8 and 10 kbp) and *Hind* III (approximately 1 and 4 kbp) digested lanes. The fact that only two bands were seen in the *Bam*H I digested lane and not the predicted three may be due to the small size of the homologous fragment produced by digestion at the first site, position 44 nt. The

Figure 4.14

Map of *Bam*H I and *Hind* III restriction sites present in *vmc*-2F1-R1 genomic PCR fragment used as a probe in the *vmc*-2 Southern analysis. As can be seen there are two sites for *Bam*H I (positions 44 and 811), one site for *Hind* III (position 434) and no sites for *Eco*R I in the above PCR fragment.

Figure 4.14 Restriction map of *vmc-2F1-R1* genomic fragment.

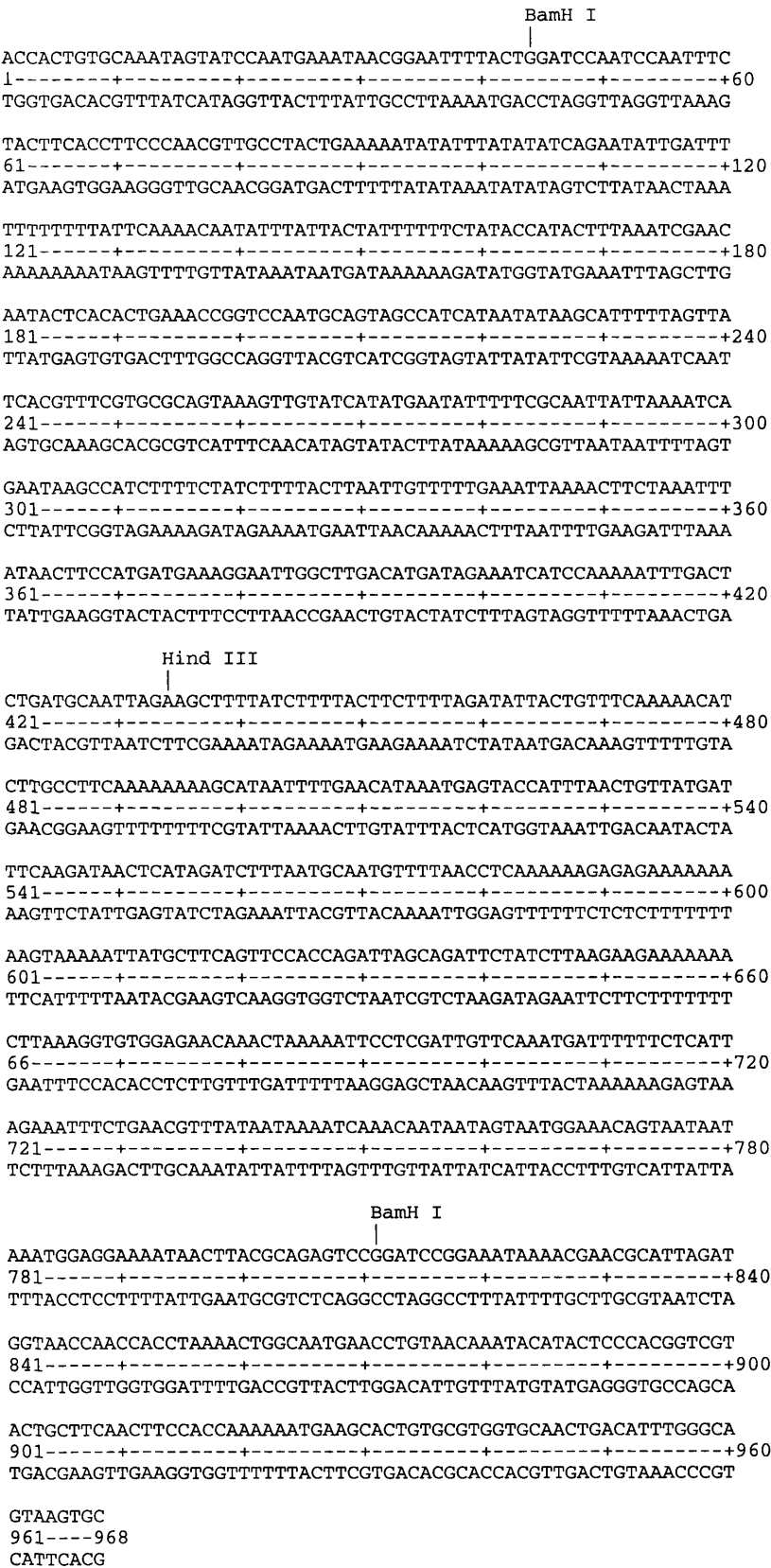


Figure 4.15a

20 µg of HMW DNA digested (16 hours, 37°C) with each restriction enzyme. Digested DNA separated on a 0.8% agarose gel.

A = DNA digested with *EcoR* I.

B = DNA digested with *BamH* I.

C = DNA digested with *Hind* III.

λH3 = *Hind* III digested lambda DNA size markers.

Figure 4.15b

Figure 4.15a blot probed with *vmc*-2F1-R1 genomic DNA probe. Washed at 50°C as follows: 4 x 10 minute 2x SSC, 0.1% SDS. The resulting autograph was exposed for 72 hours.

Figure 4.15a Ethidium bromide stained *vmc-2* Southern blot.

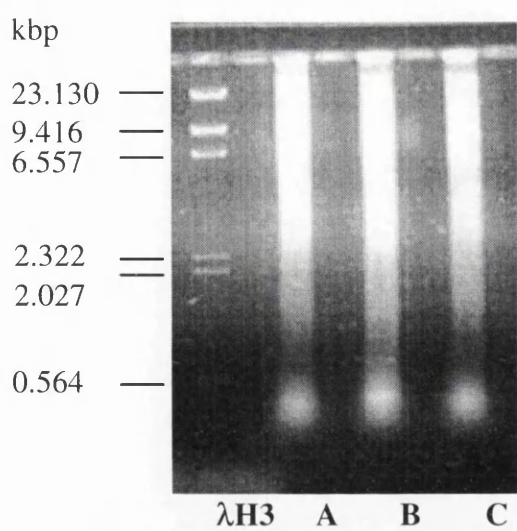
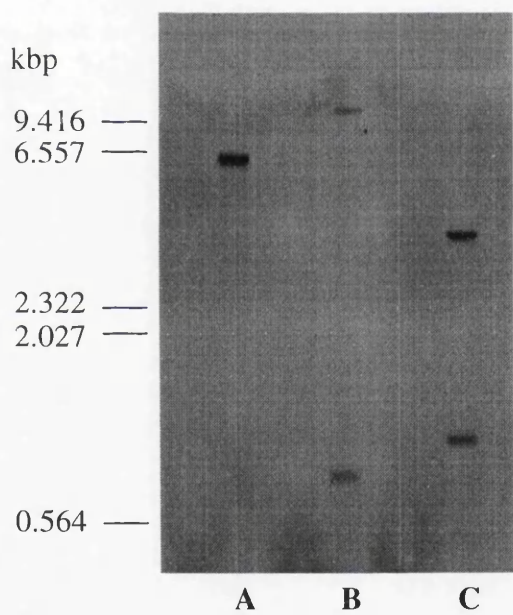


Figure 4.15b *vmc-2* Southern blot low stringency wash.



overall low hybridisation of the probe to the *Bam*H I digested DNA compared to the other lanes and the small portion of the probe that would hybridise may account for the lack of the third band. The results suggest that *vmc-2* is a single copy gene, and not a member of a larger gene family.

4.2.7 The temporal expression pattern of *vmc-2* *in vivo*.

For reasons outlined previously it was necessary to use *in vitro* derived material for screening the cDNA library. Following the library screen *vmc-2* appeared to be up-regulated in mf cultured under mosquito-like conditions. In order to determine if a similar expression pattern would be seen *in vivo* a series of experiments were conducted to investigate the expression of the *vmc-2* mRNA throughout the life-cycle of *B. pahangi* (see section 2.6).

4.2.7.1 Semi-quantitative RT-PCR.

Semi-quantitative RT-PCR determines the level of expression of a gene of interest compared to that of a constitutively expressed endogenous control gene [166]. For these studies the gene encoding β -tubulin was used as the constitutive control gene. β -tubulin was selected as it had previously been used in studies of gene expression in *Brugia* species [58, 114]. Two primers, β -*tubA* and β -*tubB* (see Table 2.1) were designed using the *B. pahangi* complete cDNA sequence, (GenBank accession M36380, [192]). The primers were designed to span an intron to allow cDNA to be distinguished from any contaminating genomic DNA. The predicted sizes of the amplified products were 318 bp for cDNA and 400 bp for genomic DNA. Figure 4.16 shows an ethidium bromide stained gel of amplified products of both first

Figure 4.16 Amplification of β -tubulin to show difference in size of cDNA and genomic DNA.

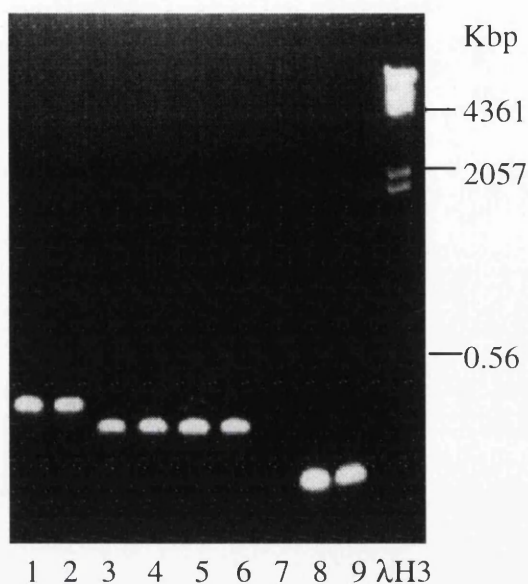


Figure 4.16

First strand cDNA from both mammalian-derived mf, vector-derived mf and *B. pahangi* genomic DNA was PCR amplified in duplicate to determine if β -tubulin could be specifically amplified using β tubA and β tubB primers as follows: Thirty cycles of: 94°C 1 minute, 55°C 1 minute, 72°C 2 minutes. Amplified products were size separated on a 1% agarose gel. A specific band of predicted size, cDNA 318bp, genomic DNA 400bp could be seen in each of the appropriate lanes.

1/2 = Genomic DNA (100 ng) β tubA- β tubB primers.

3/4 = Mammalian-derived mf cDNA (50 ng) β tubA- β tubB primers.

5/6 = Vector-derived mf cDNA (50 ng) β tubA- β tubB primers.

7 = No template DNA negative control.

8 = Mammalian-derived mf cDNA (50 ng) *mmc*-1F1-*mmc*-1R1 positive control.

9 = Vector-derived mf cDNA (50 ng) *mmc*-1F1-*mmc*-1R1 positive control.

λ H3 = *Hind* III digested λ DNA molecular size markers.

strand cDNA and genomic DNA. As can be seen single bands of the predicted size were amplified. A similar analysis was conducted using *vmc-2F1/vmc-2R1* primers. The results of this PCR are shown in Figure 4.17. The *vmc-2* primer pairs produced bands of predicted size, 252 bp and 968 bp for cDNA and genomic DNA amplifications respectively. Again this allows amplified cDNA to be distinguished from that of any contaminating genomic DNA.

4.2.7.2 Titration of gene products produced by RT-PCR.

Following a lag phase of PCR amplification cDNA products enter an exponential phase where amplicons accumulate in an exponential manner, whereas during later rounds the rate of amplification slows resulting in a plateau effect [193]. In order to compare the differences in amplification of the gene of interest and control gene products, the PCR reaction must be stopped while it is still in the exponential stage of amplification. To determine the optimal number of cycles for each of the primers sets used, 100 μ l PCR reactions were produced in the standard manner for *vmc-2* and β -*tubulin*. 10, 15, 20, 25, 30, and 35 cycles of PCR were conducted under standard conditions in a Perkin Elmer 480 thermal cycler, 10 μ l of the reaction was removed, soaked at 72°C for 10 minutes to allow complete amplification, and these products were then separated by gel electrophoresis and blotted onto nylon membrane. The resulting blots were probed with gene-specific probes and the amount of amplification was determined by scintillation counting of the specific band (section 2.6.3). The resulting autoradiographs are shown in Figure 4.18. From the graphical representation of this data (Figure 4.19), 23 cycles of PCR

Figure 4.17 Genomic DNA and cDNA PCR of and *vmc-2*.

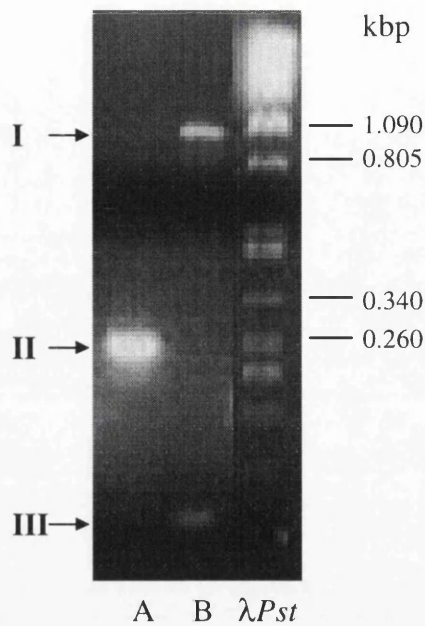


Figure 4.17
Comparison of product sizes obtained by PCR of first strand cDNA and genomic DNA, using *vmc-2* F1-R1 primers. 50ng of mammalian-derived mf first strand cDNA was PCR amplified using the appropriate primers as follows: thirty cycles of: 94°C 1 minute, 60°C 1 minute, 72°C 3 minutes. Amplified products size separated on a 1 % agarose gel. The cDNA reaction produced a band of predicted size, 250 bp (marked II) the band marked III is thought to be due to “primer-dimer” formations due to annealing of primers, the genomic reaction produced a band of approximately 1kb (marked I). The primers F1 and R1 could therefore be used in the RT-PCR reactions as the amplified cDNA products could be distinguished from any contaminating genomic DNA.

A = Mammalian-derived mf cDNA (50 ng) *vmc-2*F1-*vmc-2*R1 primers.
B = Genomic DNA (100 ng) *vmc-2*F1-*vmc-2*R1 primers.
λPst = *Pst* I digested λ DNA molecular size markers.

Figure 4.18 Titration of RT-PCR reactions.

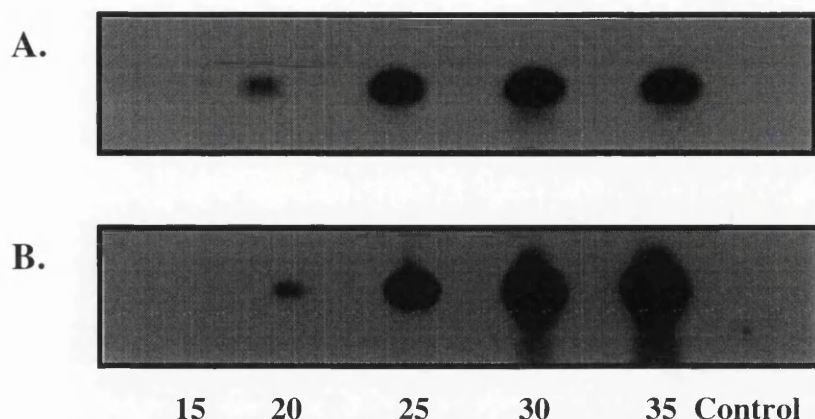


Figure 4.18

Autoradiographs showing titration of PCR amplification *vmc-2F1-R1* and β -*tubulinA-B* primer pairs on mammalian-derived mf first strand cDNA. A 100 μ l PCR was conducted under the following conditions: n cycles of: 94°C 1 minute, 55°C 1 minute, 72°C 1 minute. After each time point indicated 12 μ l of each reaction was removed and soaked at 72°C for 10 minutes to allow complete extension of products. 10 μ l from each time point was then electrophoresed on a 1 % agarose gel. The amplified products were transferred to nylon membrane and probed with a corresponding gene specific probe. The resulting Southern blots were washed at 65°C as follows: 2 x 10 minutes 2x SSC 0.1% SDS, 2 x 10 minutes 1x SSC 0.1% SDS, 2 x 10 minutes 0.1x SSC 0.1% SDS. The amount of amplified product at each time point was determined by scintillation counting of radiolabelled products. The control lane shows no DNA template control after 35 cycles of amplification.

A = *vmc-2F1-R1* amplified products.

B = β -*tubulinA-B* amplified products.

Figure 4.19 Titration of RT-PCR reaction.

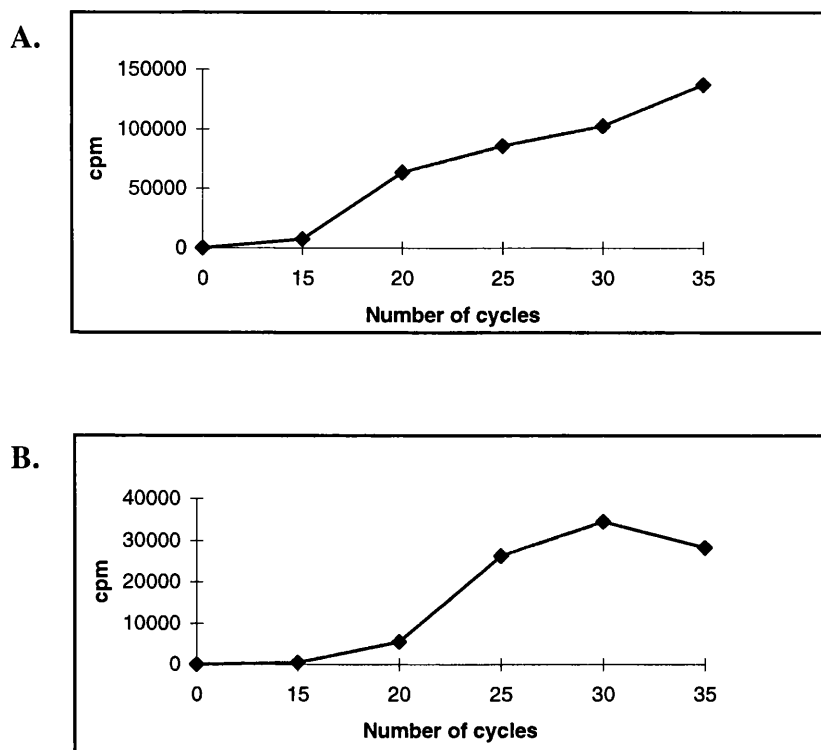


Figure 4.19

Graphical representation of data shown in Figure 4.18 Amplification of PCR products is shown as the incorporated cpm versus the number of cycles. From the graphs 23 cycles was chosen as it is in the initial phase of exponential amplification for both of the primer pairs.

A = *vmc-2* PCR

B = β -*tubulin* PCR

was chosen as this was in the early exponential stage of amplification for both *vmc-2* and the control gene β -*tubulin*.

4.2.7.3 Test of life-cycle stage cDNAs

First strand cDNA synthesis was conducted using 2 μ g of total RNA from each life-cycle stage. As a test of the first strand cDNAs each was amplified by a standard hot start PCR technique for 23 cycles using β -*tubA* and β -*tubB* primers. The resulting amplified cDNAs were separated on a 1 % agarose gel. Figure 4.20 shows an example of a test PCR. A specific band of predicted size 318 bp could be seen in each of the test lanes A-L with the exception of lane B, the uninfected mosquito control. Lane B shows a smear of products after 35 cycles of PCR presumably due to non-specific priming of related cDNAs in the mosquito. This smear does not specifically hybridise to the *B. pahangi* β -*tubulin* probe (Figure 4.21 panel B). This experiment shows that it is possible to amplify *B. pahangi* cDNA from a pool of mixed first strand cDNA isolated from infected mosquito thoraces.

4.2.7.4 Semi-Quantitative RT-PCR of *vmc-2*.

Three separate experiments were conducted to investigate expression of *vmc-2* as described above. In each case the amounts of hybridising radiolabelled *vmc-2*F1-R1 probe was calculated as described in section 2.6.3. Figure 4.21 shows an example of a *vmc-2* RT-PCR autoradiograph. The amplification of two bands in the *in vitro* vector-derived mf, lane M Figure 4.21 was unexpected, and may be due to an alternate transcript in the vector-derived mf although there is no evidence of this in the other lanes. To determine the amount of expression only the filter that

Figure 4.20 Test of β -tubulin RT-PCR on life-cycle cDNA panel.

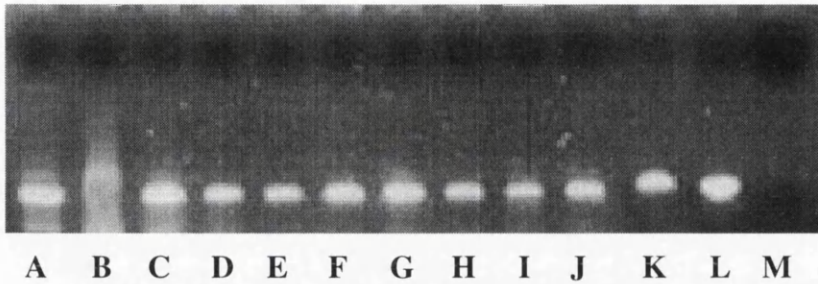


Figure 4.20

A panel of cDNAs produced from various time points throughout the parasite life cycle was tested by PCR using primers β tubA and β tubB which are specific for the constitutive control gene β -tubulin. PCR conditions as follows: Thirty cycles of: 94°C 1 minute, 62°C 1 minute, 72°C 1 minute. Amplified products were size separated on a 1 % agarose gel. A specific band of predicted size, cDNA 318bp. Could be seen in each of test lanes A-L with the exception of lane B, the uninfected mosquito control.

A = Mature mf.

B = Uninfected mosquito thorax.

C = Mosquito thorax 24 hour post infection (mf).

D = Mosquito thorax 3 days post infection (sausage stage larvae)

E = Mosquito thorax 5 days post infection (L2).

F = Mosquito thorax 8 days post infection (L3).

G =Infective L3 isolated from mosquito into Grace's medium.

H = 24 post infection, mammal (L3).

I = 3 day post infection, mammal (L3).

J = 5 day post infection, mammal (L3).

K = 10 day post infection, mammal (L4).

L = Adults worms.

M = No template control.

Figure 4.21 *vmc-2* life-cycle panel semi-quantitative RT-PCR.

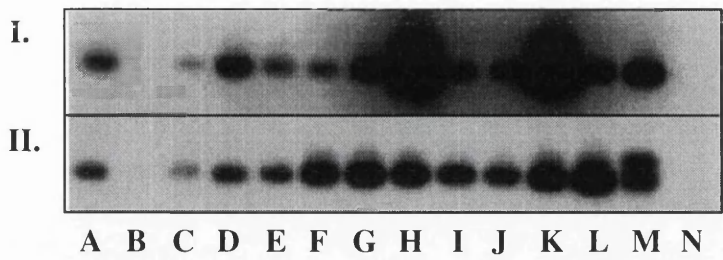


Figure 4.21

First strand cDNA amplified using either β tubA and β tubB primers (panel I) or *vmc-2F1* and *vmc-2R1* primers (panel II) under the following conditions: Twenty three cycles of: 94°C 1 minute, 62°C 1 minute, 72°C 1 minute. Amplified products were size separated on a 1 % agarose gel and transferred to nylon membrane. The resulting blots were probed with the corresponding gene specific probe under high stringency (65°C, washed to 0.1 x SSC 0.1 % SDS) and exposed to autoradiographic film. The relative expression of *vmc-2* compared to that of β -*tubulin* at each life-cycle stage was then compared by scintillation counting.

Panel I = β -*tubulin* RT- PCR.

Panel II = *vmc-2* RT-PCR.

A = Mature mf.

B = Uninfected mosquito thorax.

C = Mosquito thorax 24 hour post infection (mf).

D = Mosquito thorax 3 days post infection (sausage stage larvae)

E = Mosquito thorax 5 days post infection (L2).

F = Mosquito thorax 8 days post infection (L3).

G = pre-infective L3 isolated from mosquito into Grace's medium.

H = 24 post infection, mammal (L3).

I = 5 day post infection, mammal (L3).

J = 10 day post infection, mammal (L4).

K = Adults worms.

L = *In vitro* mammalian-derived mf cDNA.

M = *In vitro* vector-derived mf cDNA.

N = No template control.

corresponded to the band of 250 bp was removed and counted. The results of the expression analysis are given in Table 4.1, Figure 4.22 shows the average of the three RT-PCR experiments in a graphical form. As can be seen *vmc-2* expression is very low in the mf and then increases during development of the parasite in the mosquito vector. The level of expression increases by day 5 and peaks at day 8 p.i. The expression decreases slightly in the vector-derived L3 and drops very rapidly by 24 hours p.i of the mammal. The level of expression increases again at day 5 p.i (p.i. L3) and day 10 (L4), but drops dramatically in the adult parasite. Also included in the RT-PCR experiments were the first strand cDNAs produced by the *in vitro* culture methods described in Chapter 3. If the results from the mf stage *in vitro* and *in vivo* are compared it is clear that the *in vitro* material shows a difference between the mammalian and vector-derived mf that is not observed with *in vivo* derived material. The level of expression is approximately doubled in the vector compared to the mammalian-derived mf *in vitro* (5.38 and 2.83 mean cpm respectively, Table 4.1), whereas *in vivo* the mRNA abundance of mf and 24 hour mosquito samples are essentially equal (0.52 and 0.47 mean cpm respectively, Table 4.1).

As the expression level appears to drop radically from day 10 (L4) to the adult stage, the level of expression in the days prior to the L4-adult moult was also investigated. In *B. pahangi* the fourth moult (L4-Adult) is not synchronous between sexes, males moult at approximately day 23 p.i whereas the females moult between days 27 and 33 p.i. [7]. To study the expression of *vmc-2* during the late L4 stage a panel of cDNAs produced from worms at days 18, 19, 20, 21 and 22 p.i. was obtained (provided by Sarah Hunter, University of Glasgow). Semi-quantitative RT-PCR was conducted on two separate occasions under the same conditions described

Table 4.1 *vmc-2* life-cycle RT-PCR scintillation counts.

Exp #1	mf	uninfected mosquito	24 hr p.i	3 day p.i	5 day p.i	8 day p.i	Grace's L3	24 hr p.i mammal	5 day p.i	10 day p.i	Adult	mammalian-derived mf	vector-derived mf	no template DNA control
<i>B-tubulin</i>	736	350	512	1563	663	679	1807	46750	1050	939	53151	871	1781	266
<i>vmc-2</i>	524	215	309	717	961	1269	3667	3969	1323	836	6603	4708	12910	227
<i>B-tubulin</i> minus control	470	84	246	1297	397	413	1541	46484	784	673	52885	605	1515	-
<i>vmc-2</i> minus control	297	-12	82	490	734	1042	3440	3742	1096	609	6376	4481	12683	-
<i>vmc-2/B-tubulin</i>	0.63	-0.14	0.33	0.38	1.85	2.52	2.23	0.08	1.40	0.90	0.12	7.41	8.37	-

exp #2	mf	uninfected mosquito	24 hr p.i	3 day p.i	5 day p.i	8 day p.i	Grace's L3	24 hr p.i mammal	5 day p.i	10 day p.i	Adult	mammalian-derived mf	vector-derived mf	n o template DNA control
<i>B-tubulin</i>	325	241	292	405	295	383	543	9319	428	424	16753	788	435	194
<i>vmc-2</i>	237	205	221	258	336	404	610	614	320	372	666	436	1108	280
<i>B-tubulin</i> minus control	122	38	89	202	92	180	340	9116	225	221	16550	585	232	-
<i>vmc-2</i> minus control	39	7	23	60	138	206	412	416	122	174	468	238	910	-
<i>vmc-2/B-tubulin</i>	0.32	0.18	0.26	0.30	1.50	1.14	1.21	0.05	0.54	0.79	0.03	0.41	3.92	-

exp #3	mf	uninfected mosquito	24 hr p.i	3 day p.i	5 day p.i	8 day p.i	Grace's L3	24 hr p.i mammal	5 day p.i	10 day p.i	Adult	mammalian-derived mf	vector-derived mf	n o template DNA control
<i>B-tubulin</i>	516	232	285	743	417	354	865	23760	590	612	23942	1352	756	192
<i>vmc-2</i>	412	227	294	512	430	832	1137	1078	352	616	1350	987	2390	219
<i>B-tubulin</i> minus control	324	40	93	551	225	162	673	23568	398	420	23750	1160	564	-
<i>vmc-2</i> minus control	193	8	75	293	211	613	918	859	133	397	1131	768	2171	-
<i>vmc-2/B-tubulin</i>	0.60	0.20	0.81	0.53	0.94	3.78	1.36	0.04	0.33	0.95	0.05	0.66	3.85	-

Mean <i>vmc-2/B-tubulin</i>	0.52	0.08	0.47	0.40	1.43	2.48	1.60	0.05	0.76	0.88	0.07	2.83	5.38
Standard deviations	0.171	0.194	0.297	0.119	0.460	1.320	0.551	0.023	0.564	0.082	0.049	3.970	2.590

Table 4.1

Raw data obtained from triplicate *vmc-2* RT-PCR experiments using cDNAs from throughout the life-cycle. All data is expressed in CPM as determined by scintillation counting of *vmc-2* and β -*tubulin* hybridising cDNA.

Figure 4.22 Relative levels of *vmc-2* expression during the *B.pahangi* life-cycle.

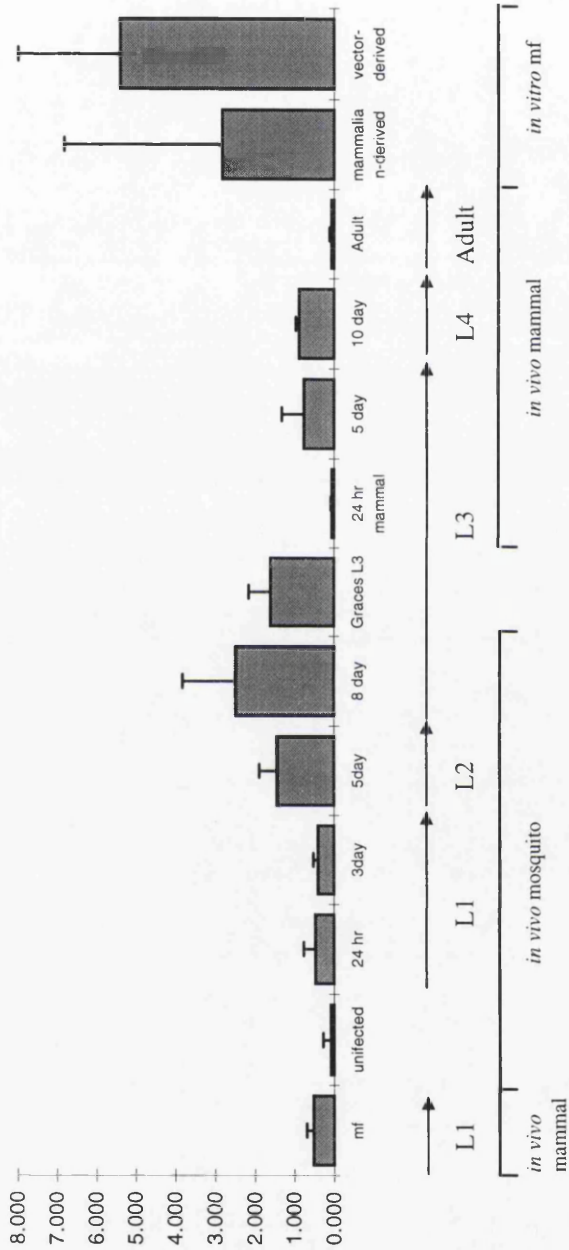


Figure 4.22

The graph shows the relative levels of *vmc-2* expression throughout the life cycle as compared to that of β -*tubulin*. The data represents the mean values obtained from three semi-quantitative RT-PCR experiments as shown in Table 4.2. The error bars represent the standard deviations from, the mean of the data from the three experiments. The levels of expression of *vmc-2*/ β -*tubulin* are in arbitrary units. The figure also shows the stage of development and the source of the material for production of the staged cDNAs.

above. Figure 4.23 and Table 4.2 shows the data obtained from these experiments. The levels of expression rise from day 18 to day 19 p.i. then remain relatively stable until day 22 p.i. where a reduction in the level of expression can be seen.

4.3 Discussion.

As shown in this chapter the gene *vmc-2* has a potential homologue in the *C. elegans* genome. Comparison of the peptide sequences of *vmc-2* and T05H10.7 showed 44 % identity over the 79 amino acids of the *vmc-2* ORF. It is encouraging that the homology extended over the entire length of the ORF and was not restricted to a small portion of the peptide. As *vmc-2* is known to be a truncated fragment of a gene perhaps if the full length sequence were available then a greater degree of homology would be seen. The lack of homology at the nucleotide level is likely to be due to the differing codon bias between the two nematode species and to third position degeneracy; because of these factors it is more appropriate to rely on comparison of the amino acid sequences when comparing genes that are not closely homologous, and more distant relations are sought. It is unfortunate that T05H10.7 is a predicted protein and no annotation exists that may hint at a possible function for the protein. Without this kind of evidence the amino acid sequences were used to scan the Pfam database to try to detect a domain or structural property that may allow the inference of function. The program found a similarity between *vmc-2* and T05H10.7 to the PfamB 35977 domain family. Members of the family that had a determined function were the GLP genes.

If the *vmc-2* peptide sequence is compared to that of the *E. coli* GLP-Q gene (accession number P09394) it shows 28.6 % identity but only over a small region of

Table 4.2 *vmc-2* late panel RT-PCR scintillation counts.

exp #1	day 18	day 19	day 20	day 21	day 22	No template control
<i>B-tubulin</i>	42112	65503	76418	64089	65065	637
<i>vmc-2</i>	9370	13503	16266	14893	5947	256
B-tubulin -control	41475	64866	75781	63452	64428	
<i>vmc-2</i> -control	9114	13247	16010	14637	5691	
<i>vmc-2</i> /B-tubulin	0.22	0.204	0.211	0.231	0.088	

exp #2	day 18	day 19	day 20	day 21	day 22	No template control
<i>B-tubulin</i>	92309	54523	69003	57220	44557	558
<i>vmc-2</i>	16967	29683	29895	25565	8847	242
B-tubulin -control	91751	54523	69003	57220	44557	-
<i>vmc-2</i> -control	16725	29683	29895	25565	8847	-
<i>vmc-2</i> /B-tubulin	0.182	0.544	0.433	0.447	0.199	-

	day 18	day 19	day 20	day 21	day 22
Mean <i>vmc-2</i> /B-tubulin	0.201	0.374	0.322	0.339	0.143
standard deviation	0.026	0.241	0.157	0.153	0.078

Table 4.2

Raw data obtained from duplicate *vmc-2* RT-PCR experiments using cDNAs from days 18-22 post infection, (late L4). All data is expressed in CPM as determined by scintillation counting of *vmc-2* and β -*tubulin* hybridising cDNA.

Figure 4.23 Relative levels of *vmc-2* expression during days 18-22 p.i. (late L4).

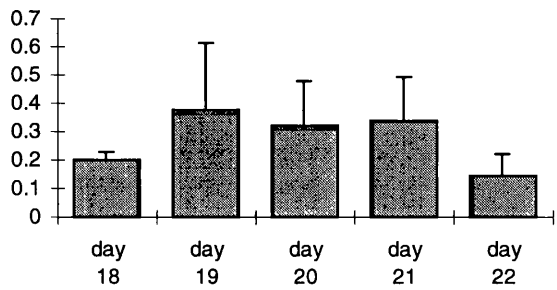


Figure 4.23

The graph shows the relative levels of *vmc-2* expression during the late L4 stage of the parasite as compared to that of β -*tubulin*. The data represents the mean values obtained from two semi-quantitative RT-PCR experiments as shown in Table 4.3. The error bars represent the standard deviations from, the mean of the data from the duplicate experiments. The levels of expression of *vmc-2*/ β -*tubulin* are in arbitrary units. As can be seen *vmc-2* expression increases slightly from day 18 to day 19 p.i. remains relatively constant during days 19-21 and drops after day 21.

35 amino acids. This result is unlikely to be due to the comparison of prokaryotic and eukaryotic sequences because if the *vmc-2* sequence is compared to the *C. elegans* GLP like sequence (accession number Q20816) then the similarity is reduced to 33.3 % over a small region of 12 amino acids, whereas the comparison of *E. coli* and *C. elegans* GLP sequences shows 22.4 % identity over a much larger fragment of 246 amino acids. The Pfam program identifies regions of similarity from gene families and then tries to match a query to any of these domains. Pfam can highlight two types of homology, that to a Pfam-A domain and a Pfam-B domain. The Pfam-A domains are designed from experimental data and are therefore more robust, while the Pfam-B domains are produced by the computer program "Domainer" and are therefore theoretical domains, and contain no annotation regarding the construction of the domain sequence signature. The fact that the T05H10.7 sequence was homologous to a Pfam-B domain (Pfam-B 35977) may explain the lack of homology of T05H10.7 and *vmc-2* to the true GLP genes. Therefore *vmc-2* and the gene T05H10 are likely to share homology to each other, although it is unlikely that these two genes are true GLPs based only on the sequence alignments. A *C. elegans* cDNA clone yk24e8.5, was identified that aligned to the 5' end of the T05H10.7 clone. The cDNA clone yk24e8.5 showed 48% identity to *vmc-2* at the amino acid level and therefore may represent a homologous gene to *vmc-2*.

The length of the *vmc-2* transcript was estimated by northern blot to be approximately 600bp, which if translated, would encode a maximum of 200 amino acids. An approximation of the molecular weight of a protein can be calculated by multiplying the number of amino acids by 110, using this technique the full length transcript of *vmc-2* could only encode a protein of approximately 22 kDa. *vmc-2* is

therefore unlikely to be a strict homologue of a T05H10.7 which encodes a predicted 90.8 kDa protein, but may share some homologous domain. Alternatively if the Gene Finder programme used to determine the coding regions of the *C. elegans* genome, did not identify the end of the gene producing the yk24e8.5 EST or the start of another gene, then as T05H10.7 is a hypothetical protein it may not encode a single protein of 90.8kDa, but several genes, one of which (yk24e8.5) is homologous to *vmc-2*. This is based on the fact that the Gene Finder program is not infallible and may miss the 5' start site of genes especially at the overlapping ends of two cosmids, which is where yk24e8.5 lies. Unfortunately there are no ESTs corresponding to *vmc-2* in the *B. malayi* dataset.

Analysis of the genomic organisation of the *vmc-2* fragment produced a number of interesting findings. A single intron of length 716 bp is relatively rare in *Brugia* species. Hammond (1994) analysed 7 genomic clones from *Brugia* species and found introns ranging in size from 64 to 1115 nucleotides, with the majority in the size range 100-149 nucleotides [194]. Similarly Zang *et al* (1999) analysed the 86 introns of all 13 *B. malayi* genomic clones currently characterised and found that only 5.8 % were in the size class 500-1000 nucleotides [128]. The possible importance of such a large intron is not known. There are instances in plants and animals where intronic sequences can influence the levels of expression of certain genes [195, 196], including the *unc-54* gene of *C. elegans* [197]. However this is not thought to be related to size of the intron but to a small enhancer region within the non-coding region. The intron/exon boundaries conformed to the general rule of exon/GT..AG/exon [198]. Also the 3' splice site showed the extended conserved

consensus sequence TTTCAG in which the -5 position (underlined) is T in 93 % of the *B. malayi* cases as described by Zang et al (1999) [128].

Truncation of the *vmc-2* gene fragment isolated from the mammalian-derived mf library seems to be a problem inherent in the techniques involved in this kind of study and has been reported previously [77, 114, 154]. As stated earlier the truncation at the 3' end of the cDNAs used to produce the library was likely to be caused by the annealing of the oligo (dT) primer to adenine rich regions in the mRNA. This may be due to the particularly high AT composition of the *Brugia* genome. The bias towards AT richness in lower eukaryotes is particularly evident in intron compared to exon sequences, 85 % and 59 % respectively in *Tetrahymena thermophila* [199] but this should not effect the isolation of cDNAs amplified from mature mRNAs where only exonic sequences are amplified. However when Zang et al (1999) analysed the percentage A+T composition of all the *B. malayi* genes to date where the full length genomic sequence was known, the A+T composition was found to be 62 % and 69 % for exons and introns respectively [128]. The high A+T percentage found in the exons of *B. malayi* is likely to be mirrored in *B. pahangi* and may therefore be one of the factors impeding the isolation of full length transcripts by RT-PCR from *Brugia* species. The calculated percentage A+T of the intron of *vmc-2* is 74 % whereas the percentage A+T in the exon sequences is 57%. The mean percentage A+T for the exonic regions of *vmc-2* is therefore 57 %. This is lower than that calculated from the *B. malayi* sequences but is slightly higher than that calculated from *C. elegans* sequences where the mean percentage A+T was found to be 54 % and 70 % in exons and introns respectively [200].

In retrospect a number of factors may have hindered the various attempts to isolate the 3' end of the *vmc-2* gene, namely the low concentration of transcript and the selection of starting material used. If the results of the northern blot analysis of *mmc-1* and *vmc-2* are compared it can be seen that in the mf the abundance of *vmc-2* is relatively low compared to *mmc-1*. Northern blots using the same amount of total mf RNA (3 µg) and radioactive probes of comparable specific activity which were hybridised and washed under the same conditions, needed very different exposure times, 4 hours (*mmc-1*) and 14 days (*vmc-2*) to see a specifically hybridising band (compare Figures 4.7 and 5.14). This presumably reflects the relative abundance of each of the transcripts in the total RNA population. The second factor that was likely to have affected the success of the *vmc-2* 3' RACE techniques was the selection of material used, primarily L3 or adult parasites. This was due to the larger size of these parasite stages and the relative ease of obtaining and purifying these parasites. However both stages show a reduction in the relative abundance of *vmc-2* (Figure 4.22), which would mean that the amount of *vmc-2* mRNA was very limiting. This would also be the case for the Marathon RACE technique which was conducted on adult stage total RNA. In retrospect cDNA prepared from pre-infective L3 parasites (day 8 mosquito) would have contained a larger proportion of *vmc-2* transcript and may have optimised the chance of success.

The expression profile of *vmc-2* is interesting in that it peaks in L3 parasites prior to transmission to the mammalian host. The levels of expression in the L1 stage (mf) are relatively equal in both the mammalian and mosquito hosts (*in vivo* derived material). The expression begins to rise significantly at day 5. The L1-L2 moult occurs between days four and five [9] and so this probably represents an increase in

expression following the first moult. A further increase is seen by day 8 at which time the majority of the worms are L3 [9]. The infective L3 collected at day 9 p.i. into Grace's tissue culture medium show a slight reduction in the expression of *vmc-2*, but this drops very rapidly to almost zero in the 24 hour p.i. L3 sample. Therefore the up-regulation of *vmc-2* expression begins in the L2 and continues through the L3 until transfer to the mammalian host. This pattern of expression is reminiscent of a 23 kDa antigen of *Onchocerca volvulus* infective larvae described by Bianco *et al*, (1990) [201]. Synthesis of this protein is initiated at or following the second moult and levels of expression increase rapidly during the L3 stage, but cease between 24 and 72 hours in mammalian culture. Conversely the secretion of the protein is seen in the mammalian derived L3 in response to the temperature shift from poikilothermic to homeothermic hosts. It was proposed that such proteins may be synthesised in earlier stages (L2) in readiness for secretion following the infection event (L3). This pattern of expression is also seen in some *B. malayi* proteins where the synthesis of L3 surface molecules are initiated in the L2 stage [202]. Based on the expression pattern of *vmc-2*, the resulting protein may be involved in the infection event, or in the maintenance of the L3 developmental block prior to infection of the mammalian host, and as such is potentially very interesting. The levels of *vmc-2* expression increase in the mammalian host during the late L3 and L4 stages and drop rapidly by the adult stage. RT-PCR on late L4 stage cDNA suggests that the levels of expression remain relatively constant throughout the L4 stage and that the drop in expression follows or is concurrent with the L4-adult moult.

Due to the problems encountered in obtaining the 3' end of the *vmc-2* transcript and, without such, the difficulty of analysing this gene in any depth,

coupled with the added interest in *mmc-1*, it was considered more profitable to concentrate on the analysis of *mmc-1*.

CHAPTER FIVE

5.1 Introduction.

The results presented in Chapter 3 suggested that *mmc-1* would be up-regulated in mammalian-derived mf. The experiments undertaken in this chapter analysed the basic features of the *mmc-1* gene and its predicted protein with the aim of determining the function of the gene in the parasite life-cycle. As described in the introduction, the mf of *Brugia* are highly adapted to life in the mammalian host. *W. bancrofti* and *B. malayi* mf show a periodicity so that the numbers of mf in the peripheral bloodstream are maximised when mosquito biting is at a peak [33]. This has led to the hypothesis that the peripheral blood may be a hostile environment for the mf, therefore they only remain in the peripheral blood long enough to allow transmission.

The presence of mf in the bloodstream of an infected host is associated with a profound defect in parasite-specific proliferative responses. While the role of the mf in down-regulation of the host immune response is controversial, proliferative responses are most impaired, and most difficult to restore after chemotherapy in microfilariae positive individuals. Another interesting feature of the mf in the mammalian blood stream relates to the developmental block at this stage of the life-cycle. The mf can survive for over 100 days but can undergo no further development until transmitted to the mosquito vector.

With the long term aim of determining the role of *mmc-1* in the mf a variety of different experiments were carried out to further characterise the *mmc-1* gene. The nucleotide and predicted amino acid sequence were analysed. The genomic organisation was investigated by Southern blot techniques. The mRNA abundance was analysed by northern blot. A search for homologous genes in related parasitic

nematodes was conducted by a nematode species zooblot, and the pattern of expression through the life-cycle was examined by semi-quantitative RT-PCR.

5.2 Results.

5.2.1 The *mmc-1* clone.

Three independent clones from the *B. pahangi* mammalian mf cDNA library were sequenced on both strands to determine an accurate consensus sequence for the *mmc-1* gene. Figure 5.1 shows the consensus sequence of *mmc-1*; the length of the clone isolated, from SL1 to the start of the string of adenosine residues is 278 bp. The sequence possesses a number of potential translational start sites, but only one ATG (double underlined) leads into an extended open reading frame. The conserved nematode SL1 sequence is shown in bold, and the stop site for the predicted open reading frame is shown in bold and is also double underlined. The string of adenosine residues at the 3' end of the gene is unlikely to be the true Poly-A tail as the polyadenylation signal sequence (AATAAA) is not present upstream. Therefore this clone is likely to be a truncated sequence, caused by the internal priming of oligo (dT) in the PCR reactions used to generate the cDNA for construction of the library. Analysis of the size of the mRNA transcript by northern blot agreed with this hypothesis (section 5.2.5).

To clone the full length transcript of *mmc-1* a PCR of higher stringency was employed using first stand cDNA produced with a lower concentration of adapter primer. As with the attempts to isolate the 3' sequence of *vmc-2* by decreasing the concentration of the adapted oligo (dT) primer to 2 μ M in the first strand synthesis, it was predicted that the proportion of primer that would anneal non-specifically would

Figure 5.1 Nucleotide sequence of *mmc-1* clone from mammalian mf library.

```
1      GGTTTAATTACCCAAGTTTGAGCAAAATCATGAAATATTTTATTCTTATG
51     CTAATAGTTTTTATTGCATTTAGTGCAACCATCGCTGATGATGAAACAGA
101    TGAAACAGAAAAAGAGCCGGAAGAAAAAGATGAAAAAACTGAGACCCAGG
151    ATCGCAAAGAACGAAGTGTGCTAACAAATTATGATCCAGCGATAATGGCA
201    CCAGAAATGAACGCCGATGCTGGTTTACTCTTTCGACGTAAAATACGATC
251    GGAAGAAGGATGTTGCTGTTGTTGAATGAAAAAAAAAAAAAAAAAAAAA
```

Figure 5.1.

The consensus sequence was derived from multiple clones from the mammalian mf library. These were isolated and sequenced in both directions using T7 and M13 reverse sequencing primers. The conserved SL1 sequence is shown in bold at the 5' end of the gene. A total of twelve putative start codons are present, the codon at position 30 (double underlined) is proposed as the true translational start site as this is the start of the only extended open reading frame. The stop codon TGA at position 273, (double underlined) is the proposed stop signal sequence for this open reading frame.

decrease (section 4.2.5). This was attempted in conjunction with a high stringency PCR of the first strand cDNA where the annealing temperature was raised to 62°C. A PCR amplification protocol, using the internal *mmc*-F1 and adapter (APA) primers (Table 2.1) was conducted (30 cycles of 94°C, 1 minute; 62°C, 1 minute 72°C, 2 minutes). The PCR produced a band of approximately 300 bp (Marked I, Figure 5.2). This band was excised from the gel and directly ligated into TA cloning vector (Invitrogen). Eight transformation positive colonies were used to produce plasmid DNA and were sequenced. Figure 5.3 shows the consensus sequence of the cloned 3' end of *mmc*-1 and the full length composite sequence of *mmc*-1 from SL1 to Poly-A tail. The full length of the *mmc*-1 transcript from SL1 to the start of the Poly-A tail is 434 bp. The sequence contains the potential polyadenylation signal (AATAAA, shown in bold and double underlined) which is located 106 nucleotides 3' of the stop codon (bold and underlined). The location of the signal follows the general consensus seen in *Brugia* species [194]. The additional information obtained from the cloned 3' end does not affect the sequence of the ORF as it encodes the non-translated region 3' to the proposed termination codon of the ORF. The 81 amino acid sequence of the predicted open reading frame is shown in Figure 5.4. Analysis of the sequence by the ProtParam algorithm at the ExPASy website (www.expasy.ch/tools/protparam) showed that it encodes a peptide of 9.345 kDa with a theoretical isoelectric point (pI) of 4.46.

5.2.2 Identifying homologues of *mmc*-1.

In an attempt to identify homologues of *mmc*-1, BLAST searches were conducted using both the nucleotide sequence and the translated amino acid

Figure 5.2 PCR products produced by *mmc-1* 3' RACE.

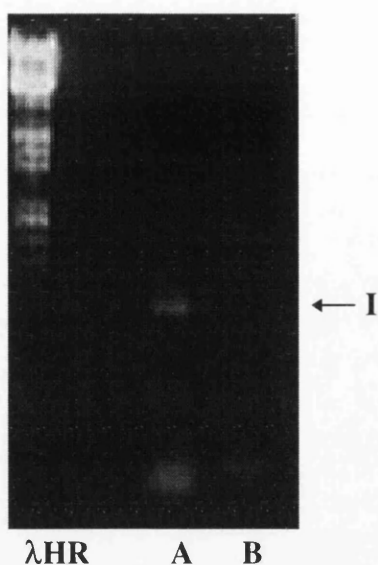


Figure 5.2

The 3' region of *mmc-1* produced by high stringency PCR using mammalian-derived mf first strand cDNA as template as follows: thirty cycles of: 94°C 1 minute, 62°C 1 minute, 72°C 2 minutes. 100 µl reaction was ethanol precipitated with 0.3 M sodium acetate. The cDNA was resuspended in 10 µl dH₂O and separated on a 0.8 % agarose gel. The band marked I was gel purified and ligated into TA vector (Invitrogen).

λHR = *Hind* III, *Eco* RI digested λ DNA molecular size markers.

A = *mmc-1*F1-APA.

B = No template control *mmc-1*F1-APA.

Figure 5.3a

Sequence of 3' end of *mmc-1* transcript obtained by PCR as shown in Figure 5.2. The PCR product was cloned into a TA vector (Invitrogen). Eight clones were sequenced in both directions by Li-cor fluorescence sequencing to produce the above consensus sequence.

Figure 5.3b

Sequence of full length transcript of *mmc-1* including the cloned 3'end of the transcript shown in Figure 5.3a. The sequence shows the SL1 spliced leader in bold the start and stop codons are shown bold, and underlined. The polyadenylation signal sequence is shown in bold and is double underlined.

Figure 5.3a Sequence of *mmc-1* 3' RACE.

1 *mmc-IF1* →
GCATTTAGTGCAACCATCGCTGATGATGAAACAGATGAAACAGAAAAAGA
51 GCCGGAAGAAAAAGATGAAAAAACTGAGACCCAGGATCGCAAAGAACGAA
101 GTGTGCTAACAAATTATGATCCAGCGATAATGGCACCAGAAATGAACGCC
151 GATGCTGGTTTACTCTTTTCGACGTAAAATACGATCGGAAGAAGGATGTTG
201 CTGTTGTTGAATGAAAAAAGAAGAAATATAAATAAATGTATTAGATAACA
251 TTAAAAAAGATTTTGAATGCTGAATGAATTTTGAGTATTTCTACATTCAT
301 CACTGTATCAATAATGAATAAATTAAATTCACTTTGAAAAAAAAAAAAA
351 **AAACTCGAGGTCGACGCGTAGATCTGAC**
← Adapter primer (APA)

Figure 5.3b Full length sequence of mmc-1.

1 **GGTTTAATTACCCAAGTTTGAG**CAAAATC**ATG**AAATATTTTATTCTTATG
51 CTAATAGTTTTTTATTGCATTTAGTGCAACCATCGCTGATGATGAAACAGA
101 TGAAACAGAAAAAGAGCCGGAAGAAAAAGATGAAAAAACTGAGACCCAGG
151 ATCGCAAAGAACGAAGTGTGCTAACAAATTATGATCCAGCGATAATGGCA
201 CCAGAAATGAACGCCGATGCTGGTTTACTCTTTCGACGTAAAATACGATC
251 GGAAGAAGGATGTTGCTGTTGT**TGA**ATGAAAAAGAAGAAATATAAATAA
301 ATGTATTAGATAACATTAAAAAGATTTTGAATGCTGAATGAATTTTGAG
351 TATTTCTACATTCATCACTGTATCAATAATG**AATAAA**TTAAATTCACCTT
401 GAAAAAAAAAAAAAAAAAAAA

Figure 5.4 Predicted amino acid sequence of *mmc-1* ORF.

```
1      MKYFILMLIVFIAFSATIADDETDETEKEPEEKDEKTETQDRKERSVLTN
51     YDPAIMAPEMNADAGLLFRRKIRSEEGCCCC*
```

Figure 5.4.

The proposed amino acid sequence, generated by translating the nucleotide sequence from the ATG at position 30, to stop codon at position 273 shown in Figure 5.3. The predicted protein consists of eighty one amino acids.

sequence. The sequence showed significant homology at the nucleotide or amino acid levels to the *B. pahangi* mRNA sequence *BpcDNA5* (accession number X95664, 99 % identity at the nucleotide level) which was previously cloned in this laboratory as part of a study to identify genes up-regulated in heat-shocked mf. *mmc-1* and *BpcDNA5* differ by a single amino acid change at the 3' end of the sequence. In the *BpcDNA5* sequence this leads to a stop codon which may be due to a sequencing error. When the *mmc-1* sequence was used to search the *C. elegans* database no significant matches were found to cosmid or EST sequences. However three homologous sequences were found in the *B. malayi* EST database (accession numbers AA228202, AA280479 and N41076). Figure 5.5 shows a ClustalW alignment of two ESTs AA228202, AA280479 and *mmc-1*. Comparison of *mmc-1* with the longest length EST, AA228202, shows 97.1 % identity at the nucleotide level and 95.1 % identity at the amino acid level. Comparisons of the two predicted peptides were conducted and are shown in Table 5.1. Both ORFs are 81 amino acids long, *mmc-1* has a slightly greater mass and contains more acidic residues, both contain a predicted cleavable signal sequence of 17 amino acids and are particularly rich in glutamic acid residues.

In an attempt to identify homologous genes of known function, with low similarity to *mmc-1* a FASTA search of GenBank using the predicted amino acid sequence of *mmc-1* was conducted. The FASTA program has been found to be more robust at finding low homology matches when compared to the BLAST algorithm [203, 204]. The FASTA search produced a low significance match to Hsp90 from chicken (51 % identity over a 27 amino acid fragment, accession number A32298). However when a *B. pahangi* *hsp90* probe was used to hybridise an array of positives

Figure 5.5 ClustalW alignment of *mmc-1* and *B. malayi* ESTs.

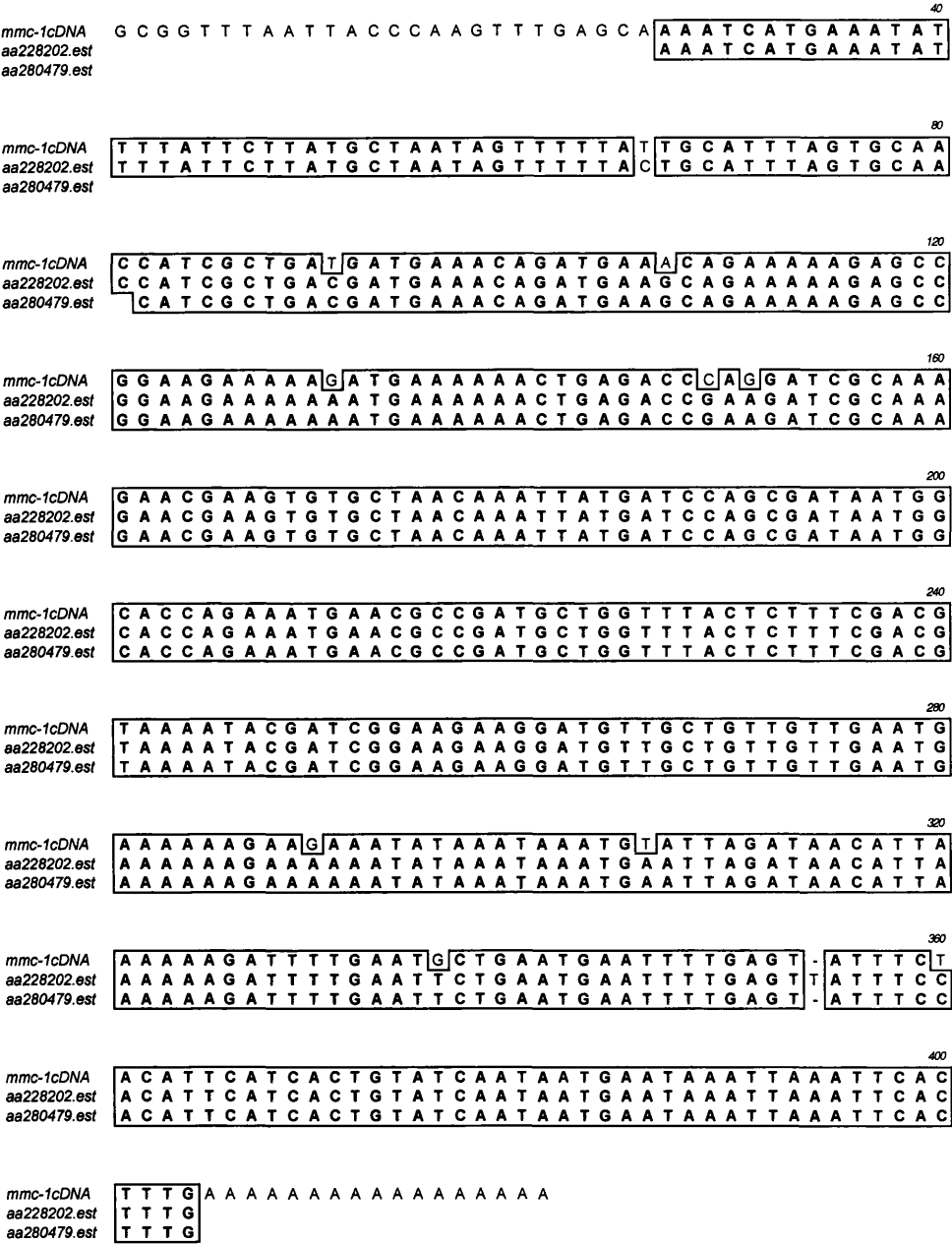


Figure 5.5
The *mmc-1* nucleotide sequence and two homologous ESTs from *B. malayi* were aligned using the ClustalW program. Matched nucleotides are shown boxed, identical nucleotides are shown in bold.

Table 5.1 Comparison of *mmc-1* ORF and the homologous *B. malayi* EST AA228202.

	<i>B. pahangi mmc-1</i>	<i>B. malayi</i> AA228202
Number of amino acids	81	81
Predicted molecular weight	9.345 kDa	9.303 kDa
Predicted pI	4.46	4.48
Percentage glutamic Acid	16 %	14.8 %
Signal Peptide	1-17 (cleavable)	1-17 (cleavable)

Table 5.1.

Parameters of the two proteins *mmc-1* and AA228202 predicted using the protparam program at the expasy website (<http://www.expasy.ch/cgi-bin/protparam>).

from the primary screen of the library, no specific hybridisation to the *mmc-1* clones was seen (Figure 3.8). The other FASTA matches were short regions of similarity to the helix-loop-helix ITF-2 transcription factors of the dog (accession number S34416) and human (accession number A41311) and a helix-loop-helix transcription factor ME2 of the mouse (accession number I52648). Each of these sequences show 30.4 % identity to *mmc-1* over a 46 amino acid fragment. Figure 5.6 shows a ClustalW line up of these three peptide sequences and the predicted *mmc-1* ORF. The line up shows a high degree of homology between the three transcription factors, whereas the *mmc-1* peptide is only homologous to the carboxyl end of the proteins and requires a number of gaps to retain this homology. A scan of the prosite database at the ExPasy website (<http://expasy.ch/prosite>) revealed a signature (PDOC00038) that is present in the three transcription factors. This signature sequence is designated the "helix-loop-helix dimerization domain" and is a requisite for the binding of the transcription factor to a DNA molecule. The boxed area of Figure 5.6 highlights this signature, it can be seen that the sequence is not present in the *mmc-1* peptide, and therefore it is unlikely that *mmc-1* is truly homologous to the helix-loop-helix transcription factor family of proteins and that the low homology is due to a forced alignment by the search program.

5.2.3 Is *mmc-1* SL1 *trans*-spliced ?

As *mmc-1* was isolated from SL1/oligo (dT) amplified cDNA it was presumed to be *trans*-spliced and therefore possess the SL1 22 nucleotide sequence at the 5' end of the transcript. In contrast the corresponding *B. malayi* ESTs AA228202, AA280479 and N41076 which were isolated from a conventional cDNA library

<i>mmc-1ORF</i>	L L S A N R H S L M V G A H R E D G V A L R G S H S L V P N Q V P V P Q L P V Q	480
<i>s34416</i>	L L S A N R H S L M V G A H R E D G V A L R G S H S L L P N Q V P V P Q L P V Q	
<i>i52648</i>	L L S A N R H S L M V G T H R E D G V A L R G S H S L L P N Q V P V P Q L P V Q	
<i>a41311</i>	L L S A N R H S L M V G T H R E D G V A L R G S H S L L P N Q V P V P Q L P V Q	
<i>mmc-1ORF</i>	M K Y F I L M L I V F I A F S A T I A D D E I T D	520
<i>s34416</i>	S A T S P D L N P P Q D P Y R G M P P G L Q G Q S V S S G S S E I K S D D E G D	
<i>i52648</i>	S A T S P D L N P P Q D P Y R G M P P G L Q G Q S V S S G S S E I K S D D E G D	
<i>a41311</i>	S A T S P D L N P P Q D P Y R G M P P G L Q G Q S V S S G S S E I K S D D E G D	
<i>mmc-1ORF</i>	E T E K E P - - - E E K - - - D E K - - - T E T Q D - - - - -	560
<i>s34416</i>	E N L Q D T K S S E D K K L D D D K K D I K S I T S N N D D E D L T P E Q K A E	
<i>i52648</i>	E N L Q D T K S S E D K K L D D D K K D I K S I T S N N D D E D L T P E Q K A E	
<i>a41311</i>	E N L Q D T K S S E D K K L D D D K K D I K S I T S N N D D E D L T P E Q K A E	
<i>mmc-1ORF</i>	R - K E R -	600
<i>s34416</i>	R E K E R R M A N N A R E R L R V R D I N E A F K E L G R M V Q L H L K S D K P	
<i>i52648</i>	R E K E R R M A N N A R E R L R V R D I N E A F K E L G R M V Q L H L K S D K P	
<i>a41311</i>	R E K E R R M A N N A R E R L R V R D I N E A F K E L G R M V Q L H L K S D K P	
<i>mmc-1ORF</i>	- - - - - - - - - - S V L T N Y D P A I M A P E M N A D A G L L F R R - - K	640
<i>s34416</i>	Q T K L L I L H Q A V A V I L S L E Q Q V R E R N L N P K A A C L K R R E E E K	
<i>i52648</i>	Q T K L L I L H Q A V A V I L S L E Q Q V R E R N L N P K A A C L K R R E E E K	
<i>a41311</i>	Q T K L L I L H Q A V A V I L S L E Q Q V R E R N L N P K A A C L K R R E E E K	
<i>mmc-1ORF</i>	I R S E E G C C C C	
<i>s34416</i>	V S S E P P P L S L A G P H P G M G D A S N H M G Q M	
<i>i52648</i>	V S S E P P P L S L A G P H P G M G D A S N H M G Q M	
<i>a41311</i>	V S S E P P P L S L A G P H P G M G D A S N H M G Q M T	

ClustalW alignment of three Helix-Loop-Helix family transcription factors that showed low homology to the *mmc-1* ORF. Similar residues are boxed, identical matches are shown in bold. The shaded box shows the H-L-H dimerisation signature.

(SAW94LS-BmMF) are reported as SL1 negative. It is likely that the two genes from such closely related parasites would both be SL1 *trans*-spliced. The difference could reflect mispriming of SL1 in the generation of the mammalian-derived mf library resulting in *mmc-1* containing a false SL1. Alternatively the sequencing protocol used to generate the *B. malayi* ESTs simply did not record the presence of the SL1.

To determine the presence or absence of the SL1 sequence 5' RACE was attempted following the manufacturers protocol as outlined in section 2.7.5. Figure 5.7 shows a schematic representation of the RACE protocol. Following first strand synthesis with a *mmc-1* gene specific primer (gsp, *mmc-1*gsp1) and dCTP tailing, PCR was conducted using a nested gsp (*mmc-1*gsp2) and an anchor primer specific to the dC tail. Thirty five cycles of PCR did not produce a clear band but did result in a smear of amplified products in the test lane (Figure 5.8, lane E). Non-specific smears of amplified products could also be seen in the control RNA lane, and dC tailed RNA control lanes (Figure 5.8 lane A and D respectively). A further round of nested PCR with *mmc-1*gsp3 and an abridged anchor primer was conducted using 5µl of a 1/100 dilution of the first round PCR products. Following thirty five rounds of PCR a faint band could be seen in the positive test lane (Figure 5.9 lane E). Again smears of products could be seen in the control lanes (Figure 5.9 lanes A-D) but not in the no template DNA control lane (Figure 5.9 lane F). An aliquot of the positive control PCR products was ligated into pCR 2.1 TA vector and transformed into "One-shot" competent cells. Transformation positive clones were digested with *Eco* RI to liberate the insert and blotted onto nylon membrane. The resulting Southern blot was probed with a *mmc-1* gene specific probe. A number of the inserts hybridised to the *mmc-1* probe (Figure 5.10) these were sequenced on both strands and a consensus sequence

Figure 5.7 Schematic diagram of RACE protocol.

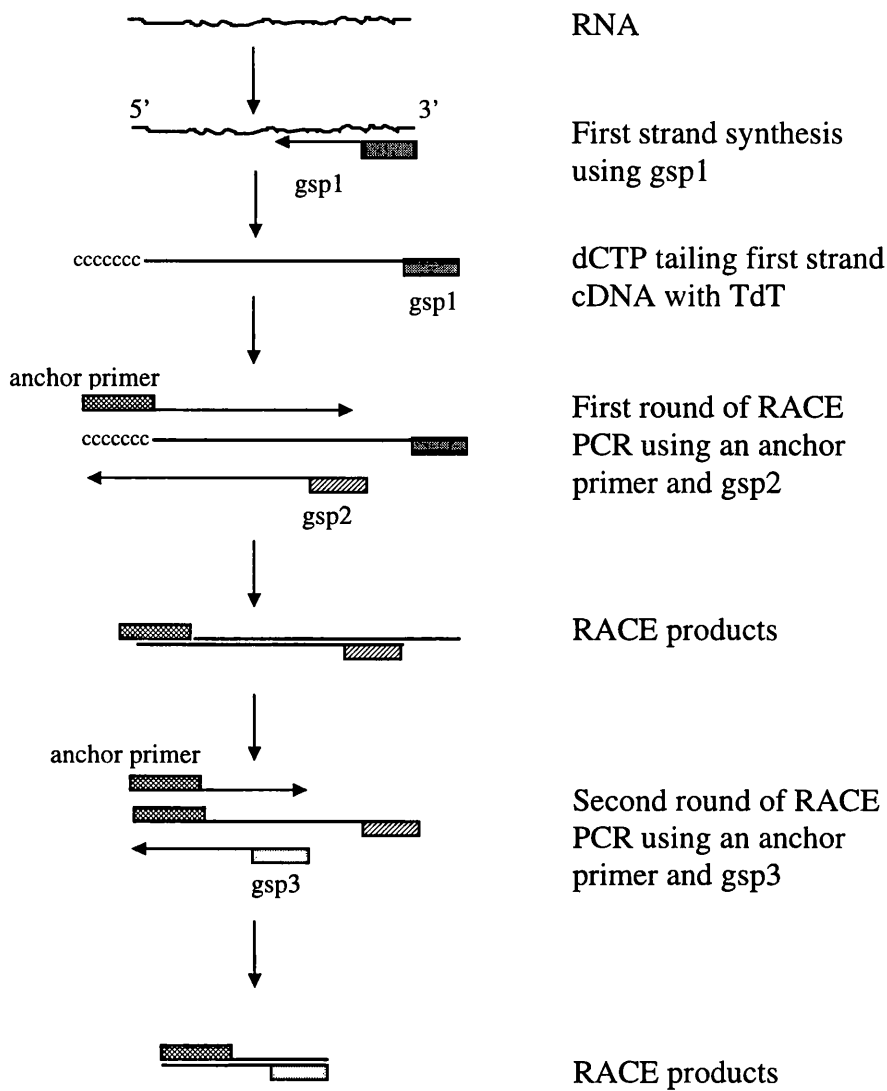


Figure 5.7
Schematic diagram showing the protocol of 5'RACE.

Figure 5.8 *mmc-1gsp2* amplified RACE products.

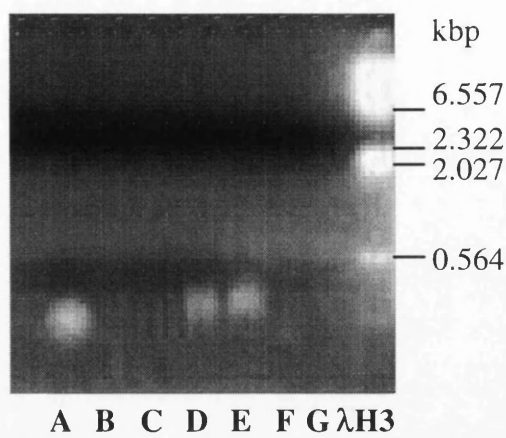


Figure 5.8

RACE amplified cDNA, all samples amplified using *mmc-1gsp2* and universal anchor primer. For thirty five cycles as follows: 94°C 1 minute, 50°C 1 minute, 72°C 2 minutes and a final extension incubation of 72°C for 10 minutes. The amplified products were size separated on a 1 % agarose gel.

A = Mammalian-derived mf RNA

B = First strand cDNA

C = Glass max purified first strand cDNA

D = dCTP tailed mammalian-derived mf RNA

E = dCTP tailed Glass max purified first strand cDNA

F = No template cDNA control

G = No primer control

λH3 = *Hind* III digested lambda DNA size markers.

Figure 5.9 *mmc-1gsp3* amplified 5' RACE products.

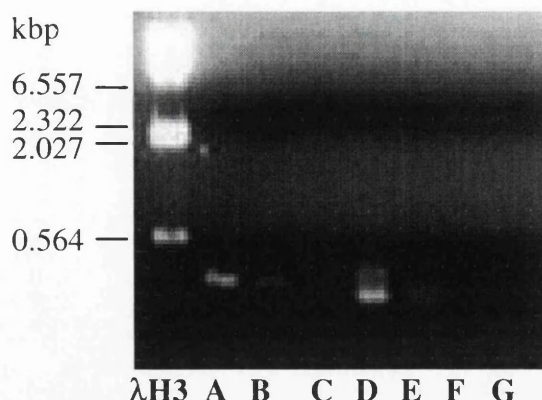


Figure 5.9

RACE amplified cDNA, all samples amplified using *mmc-1gsp3* and unabridged universal anchor primer. 5μl of a 1/100 dilution of each *mmc-1gsp2* PCR (Figure 5.8) re-amplified using a nested primer *mmc-1gsp3*. PCR conditions as follows: thirty five cycles of: 94°C 1 minute, 50°C 1 minute, 72°C 2 minutes and a final extension of 72°C for 10 minutes. The amplified products were size separated on a 1 % agarose gel.

λH3 = *Hind* III digested lambda DNA size markers

A = Mammalian-derived mf RNA

B = First strand cDNA

C = Glass max purified first strand cDNA

D = dCTP tailed mammalian-derived mf RNA

E = dCTP tailed Glass max purified first strand cDNA

F = No template cDNA control

G = No primer control

Figure 5.10 *mmc-1* 5' RACE transformation positives Southern blot.

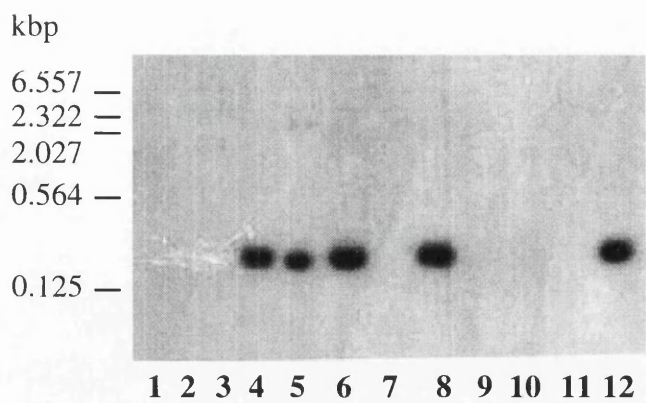


Figure 5.10
*mmc-1*gsp3/anchor primed 5' RACE cDNAs (5.9 lane E) were cloned into pCR2.1 TA vector (Invitrogen) and transformed into Oneshot competent cells. The plasmids were digested with *Eco* RI to liberate the cloned insert and analysed by Southern blot with a *mmc-1* gene specific probe. Lanes 1-12 = transformation clones.

Figure 5.11 *mmc-1* 5' RACE consensus sequence.

```
1      GGGGGGGGGGGGGGGGGGTTTAATTACCCAAGTTTGAGCAAAATCATGAAAT
51     ATTTTATTCTTATGCTAATAGTTTTTTATTGCATTTAGTGCAACCATCGCT
101    GATGA
      ← mmc-1gsp3
```

Figure 5.11
Sequence of 5' end of *mmc-1* transcript obtained by PCR as shown in Figure 5.9. The amplified PCR product was cloned into TA vector (Invitrogen). Four clones were sequenced on both strands to produce the consensus sequence shown above. The SL1 sequence is shown in bold and is underlined, the *mmc-1gsp3* primer used in the RACE protocol is also shown in bold.

of the 5' RACE was constructed. The results showed that *B. pahangi mmc-1* does possess the SL1 sequence (Figure 5.11).

5.2.4 Comparison of cDNA and genomic sequence of *mmc-1*.

In order to compare the *mmc-1* cDNA and genomic sequences, a PCR using the *mmc-1F1* and *mmc-1R1* primers was conducted using 200 ng of *B. pahangi* genomic DNA as a template. A band of approximately 1 kb (Figure 5.12) was produced that hybridised to the *mmc-1* cDNA probe. This band, named *mmc-1F1-R1* genomic was purified and cloned into a TA vector (Invitrogen). Three independent PCR clones of the *mmc-1F1-R1* genomic PCR fragment isolated as described above were sequenced on both strands. The consensus sequence is shown in Figure 5.13. A comparison of the genomic and cDNA sequences revealed the presence of two introns, shown in bold. *mmc-1* intron 1 starts at position 43, ends at position 381, and is 338 nt in length. *mmc-1* intron 2 starts at position 444, ends at position 927, and is 483 nt in length, both introns are particularly A+T rich (90 % intron 1, 79 % intron 2).

5.2.5 Northern blot analysis of *mmc-1*.

Northern blots were carried out using RNA derived from mf cultured under either mammalian or vector-derived conditions (section 4.2.4) or using mixed sex adult RNA. Blots were produced and hybridised under high stringency conditions, and exposed for 24 hours. Figure 5.14a shows an EtBr stained gel to demonstrate approximately equal loading of RNA in each lane, while Figure 5.14b shows the resulting autoradiograph. The *mmc-1* probe hybridised to a band of approximately

Figure 5.12 *mmc-1F1 mmc-1R1* genomic DNA and cDNA PCR.

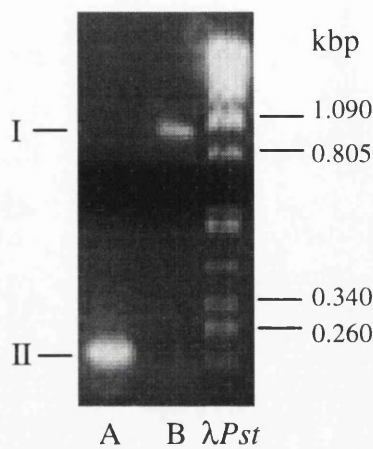


Figure 5.12

200 ng of *B. pahangi* genomic DNA was amplified by PCR using the *mmc-1F1* and *mmc-1R1* primers as follows: thirty cycles of 94°C 1 minute, 60°C 1 minute, 72°C 3 minutes. The amplified products were size separated on a 2 % agarose gel. A band of approximately 1 kb, marked I was isolated and purified. The band marked II corresponds to the cDNA control *mmc-1F1*, *mmc-1R1* control.

A = Positive control *mmc-1* plasmid PCR, *mmc-1F1* and *mmc-1R1* primers.

B = 200 ng Genomic DNA PCR *mmc-1F1-mmc-1R1* primers.

λPst = *Pst* I digested λ DNA molecular size markers.

Figure 5.13 Sequence of *mmc-1F1-R1* genomic DNA PCR fragment and position of introns.

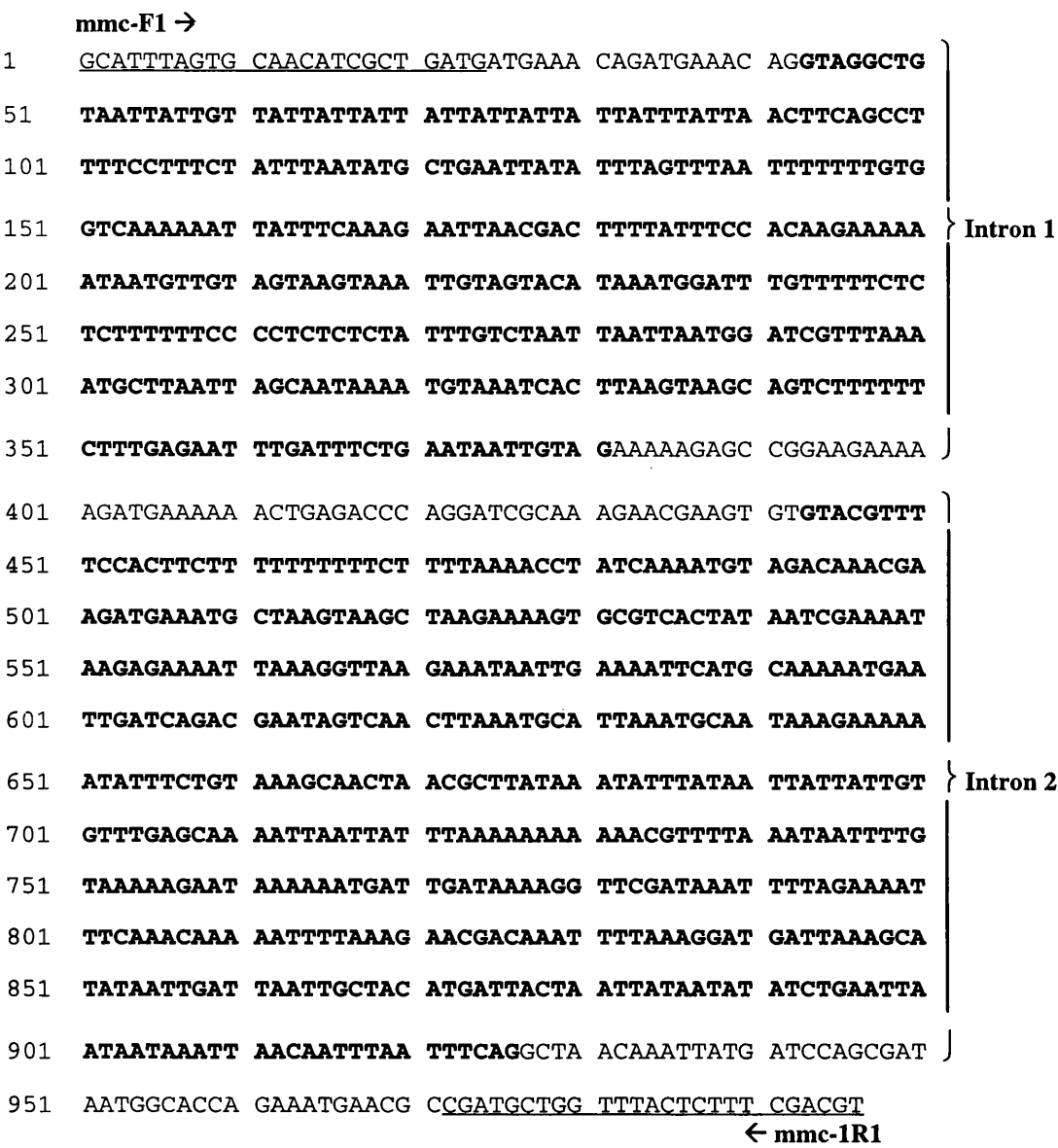


Figure 5.13
The genomic *mmc-1F1-R1* PCR product (Figure 5.12) was ligated into a TA cloning vector (Invitrogen) and sequenced on both strands on three occasions. The consensus sequence is shown above. The positions of the two primers are underlined. By comparison of the cDNA and genomic sequences two introns were detected, designated intron 1 and intron 2 (shown in bold).

Figure 5.14a

RNA was extracted using standard techniques from 2×10^6 mf cultured for 2 hours in either Grace's culture medium at 28°C (vector-derived mf) or in MEM at 37°C, (mammalian-derived mf) or from 200 mixed sex adults. Normalised samples of RNA were used for the production of the *mmc-1* northern blot. 3µg of each sample was separated on a 1.5% agarose gel containing formamide and transferred to nylon membrane for hybridisation with a *mmc-1* gene specific probe.

M = RNA markers (Gibco)

A = *in vitro* cultured vector-derived mf RNA.

B = *in vitro* cultured mammalian-derived mf RNA.

C = Mixed sex adult RNA.

Figure 5.14b

RNA samples shown in Figure 5.9a were transferred to a nylon membrane and hybridised with *mmc-1*F1-R1 cDNA probe. The blot was washed to high stringency at 65°C as follows: 2 x 10 minutes 2 x SSC 0.1 % SDS, 2 x 10 minutes 1 x SSC 0.1 % SDS, 2 x 10 minutes 0.1 x SSC 0.1 % SDS. The blot was then exposed to autoradiographic film for 24 hours at -70°C. The approximate size of the *mmc-1* transcript is 400bp (marked I).

Figure 5.14a. Ethidium bromide stained RNA.

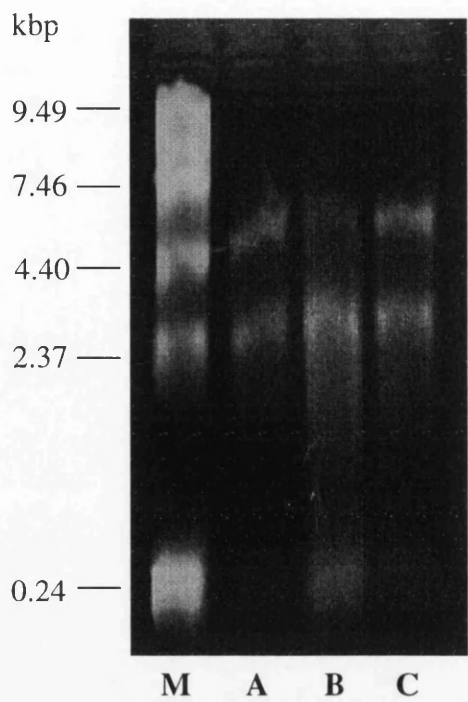
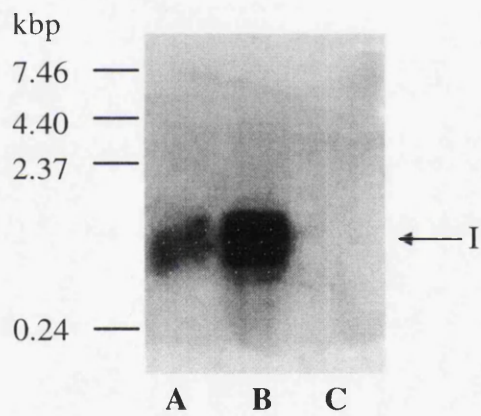


Figure 5.14b *mmc-1* northern blot.



400 bp confirming that the clone isolated from the mammalian-mf library (278 bp) was missing approximately 120 bp and the high stringency PCR protocol described above did isolate the full length clone. The intensity of the hybridisation to the three samples varied greatly. The transcript is more abundant in the mammalian, compared to the vector-derived mf (compare lanes B and A respectively), confirming the results of the differential screen. No hybridisation was observed to the adult RNA, even after extended exposure times of seven days (results not shown). As a mixed sex adult population was analysed and the adult females will contain large numbers of immature mf, this suggests that *mmc-1* is a mature mf-specific gene that is not expressed at high levels *in utero*.

5.2.6 Southern blot analysis of *mmc-1*.

To determine whether *mmc-1* is a single copy gene, or if it is a member of a larger gene family, Southern blot analysis of high molecular weight DNA was conducted. As with the Southern blot analysis of *vmc-2* (section 4.2.5), genomic DNA was isolated from approximately 300 mixed sex adult *B. pahangi* and 20 µg of DNA was digested with either *EcoR* I, *Hind* III or *Dde* I. The resulting digested DNA was electrophoresed and blotted under standard conditions. Both *EcoR* I and *Hind* III were selected as there is no restriction site for these enzymes in the *mmc-1F1-R1* PCR fragment used as a specific probe. *Dde* I has one restriction site in the *mmc-1F1-R1* fragment at position 69 bp. A map of *mmc-1F1-R1* and the position of the *Dde* I site is shown in Figure 5.15. The Southern blot was initially hybridised at low stringency with the *mmc-1F1-R1* cDNA probe. Figure 5.16 shows the EtBr stained DNA smear and the resulting autoradiograph. A number of bands can be seen in each

Figure 5.15 Restriction map of *mmc*-1F1-R1 cDNA fragment.



Figure 5.15
Map of restriction sites present in *mmc*-1F1-R1 cDNA PCR fragment used as a probe in the *mmc*-1 Southern analysis. There is one site for *Dde* I and no sites for *Hind* III or *Eco* RI in the PCR fragment.

Figure 5.16a

20 µg of HMW DNA digested (16 hours, 37°C) with each restriction enzyme. Digested DNA was separated on a 0.8% agarose gel.

A = DNA digested with *Hind* III.

B = DNA digested with *Dde* I.

C = DNA digested with *Eco* RI.

λH3 = *Hind* III digested lambda DNA size markers.

λPst = *Pst* I digested lambda DNA size markers.

Figure 5.16b.

The above Southern blot was probed with *mmc*-1F1-R1 cDNA probe. Washed at 50°C as follows: 4 x 10 minute 2x SSC, 0.1% SDS. The resulting autograph was exposed for 24 hours. Lanes as described above.

Figure 5.16a Ethidium bromide stained *mmc-1* Southern blot.

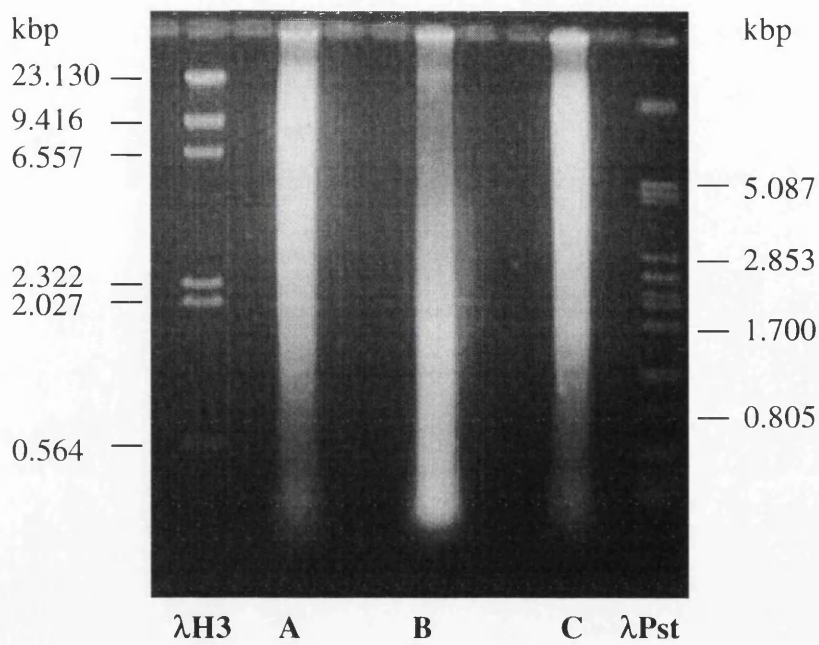
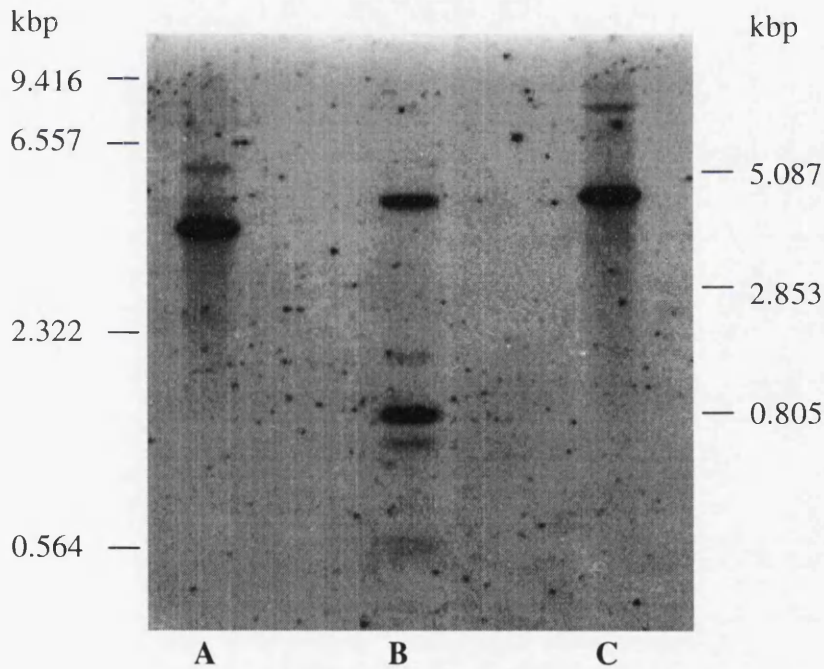


Figure 5.16b *mmc-1* Southern low stringency wash.



of the lanes. A single band of higher intensity can be seen in both the *EcoR* I and *Hind* III digested DNA lanes, and two high intensity bands in the *Dde* I lane. The low stringency of the washing and the low temperature of hybridisation may have caused cross-hybridisation to other regions of the genome. To test if this assumption was correct the same blot was washed to slightly higher stringency (50 °C, 2x 10 minutes 1 x SSC, 0.1% SDS). The autoradiograph produced after exposure for 72 hours is shown in Figure 5.17. Washing at higher stringency resulted in a single band in the *EcoR* I lane of approximately 5 kbp, a single band in the *Hind* III lane of approximately 4 kbp and two bands in the *Dde* I lane, one of approximately 4 kbp and one of approximately 1 kbp. This experiment suggests that *mmc-1* is a single copy gene, and not a member of a larger gene family.

5.2.7 Zooblot analysis of *mmc-1*.

As *mmc-1* is expressed in both *B. pahangi* and *B. malayi* species but does not show homology to genes of the free living nematode *C. elegans*, it is a potential parasite-specific gene. In order to determine if homologous sequences are present in other filarial nematodes, genomic DNA from various nematode species was analysed by low stringency Southern blot. The parasite species tested were; *Acanthocheilonema viteae*, *Onchocerca gibsoni*, *Diofilaria immitis*, *Litomosoides sigmodontis* and *Loa loa*. By comparison of filarial species that have sheathed mf (*B. pahangi*, *L. sigmodontis* and *L. loa*) and non-sheathed mf (*D. immitis*, *A. viteae*, *O. gibsoni*), it was possible to determine if the expression of *mmc-1* is correlated with the possession of the microfilarial sheath.

Figure 5.17 *mmc-1* Southern higher stringency wash.

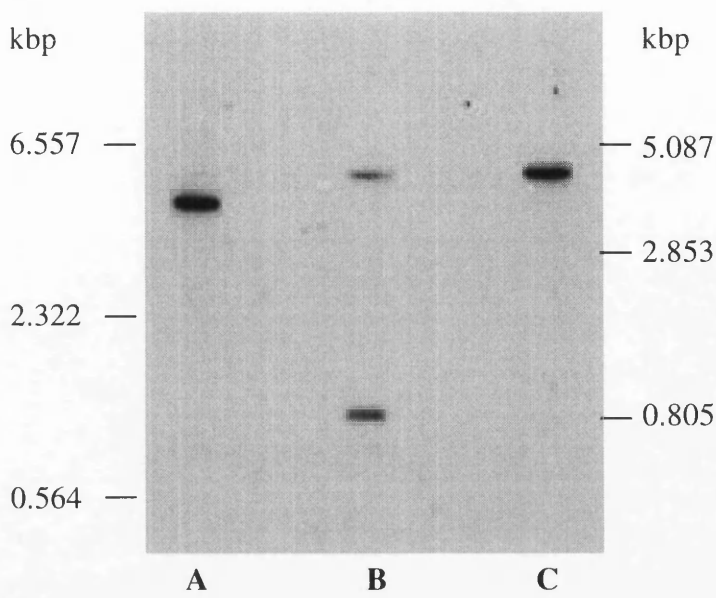


Figure 5.17.
mmc-1 Southern blot shown on figure 5.16b washed to higher stringency at 50°C as follows: 3 x 10 minutes 1 x SSC , 0.1 % SDS. The resulting autograph was exposed for 72 hours. Legend as described in Figure 5.16a.

15 µg of genomic DNA was digested using *Dde* I, electrophoresed and blotted by standard procedures. Hybridisations were conducted at 50°C and washed to low stringency. As described in 5.2.6 digestion of *B. pahangi* genomic DNA with *Dde* I produced two bands of approximately 1 and 4 kbp. The results of the Southern blot with *B. pahangi*, *A. viteae*, *D. immitis* and *O. gibsoni* DNA are shown in Figure 5.18. Hybridisation was observed only to the *B. pahangi* DNA, producing bands of predicted size (Figure 5.18b lane A), whilst the DNA from the other nematodes did not show any hybridisation.

Comparison of *L. sigmondontis* and *B. pahangi* DNA is shown in Figure 5.19. In this experiment three bands of hybridisation to the *B. pahangi* DNA were observed (Figure 5.19b lane A). This is most likely to be due to incomplete digestion of the DNA, as the larger band (approximately 5 kb) is the correct size for an undigested form of the two smaller bands. More interesting is the presence of a single band of approximately 2.3 kb (Figure 5.19b lane B, marked I) in the *L. sigmondontis* DNA. However when the Southern blot was washed to higher stringency (50°C, 2x 10 minutes 1x SSC, 0.1 % SDS) the band was no longer visible (Figure 5.19b (ii)). Similarly at low stringency a degree of cross hybridisation was seen to *L. loa* DNA (Figure 5.20). In addition to the two predicted bands in *B. pahangi* DNA, a number of weakly hybridising bands were observed. Two of the weakly hybridising bands are mirrored in the *L. loa* sample (marked I), whilst the strongly hybridising bands, characteristic of *mmc-1* of 4kb and 1 kb are not. When the Southern blot was washed to slightly higher stringency as with the *L. sigmondontis* sample, the hybridising bands were no longer visible (Figure 5.20b (ii)).

Figure 5.18a

High molecular weight DNA isolated from various filarial worms was digested with *Dde* I, electrophoresed on a 0.8 % agarose gel and visualised under UV light to show approximately equal loading of samples.

λ H3 = *Hind* III digested λ DNA molecular size markers.

A = 15 μ g of *Dde* I digested *B. pahangi* genomic DNA.

B = 15 μ g of *Dde* I digested *A. viteae* genomic DNA.

C = 15 μ g of *Dde* I digested *O. gibsoni* genomic DNA.

D = 15 μ g of *Dde* I digested *D. immitis* genomic DNA.

Figure 5.18b

The Southern blot produced from the electrophoresed material shown above was hybridised at low stringency with a *mmc*-1F1-R1 cDNA probe (50°C, washed 2 x SSC 0.1% SDS 2 x 10 minutes). The autoradiograph was exposed for 72 hours at 70°C. Lanes as shown for 5.18a.

Figure 5.18a Ethidium bromide stained filarial nematode DNA.

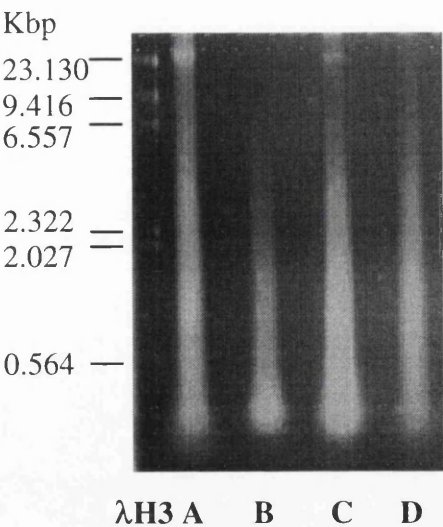


Figure 5.18b Filarial nematode *mmc-1* blot.

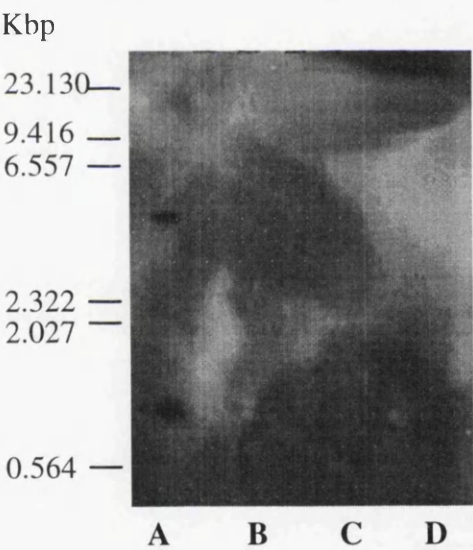


Figure 5.19a.

High molecular weight DNA isolated from *B. pahangi* and *L. sigmodontis* filarial worms was digested with *Dde* I, electrophoresed on a 0.8 % agarose gel and visualised under UV light to show approximately equal loading of samples.

λ H3 = *Hind* III digested λ DNA molecular size markers.

A = 15 μ g of *Dde* I digested *B. pahangi* genomic DNA.

B = 15 μ g of *Dde* I digested *L. sigmodontis* genomic DNA.

Figure 5.19b(i).

The Southern blot produced from the electrophoresed material shown in Figure 5.13a was hybridised at low stringency with a *mmc-1*F1-R1 cDNA probe (50°C, washed 2 X SSC 0.1% SDS 2 x 10 minutes). The autoradiograph was exposed for 72 hours at -70°C. Lanes as shown for Figure 5.19a. The cross reaction of *mmc-1* to the *L. sigmodontis* DNA in lane B after the low stringency wash is marked I.

Figure 5.19b(ii).

Higher stringency wash, the *L. sigmodontis* zooblot was washed to slightly higher stringency (50°C, washed 1 X SSC 0.1% SDS 2 x 10 minutes). The autoradiograph was exposed for 72 hours at -70°C. Lanes as shown for Figure 5.19a.

Figure 5.19a Ethidium bromide stained *B. pahangi* and *L. sigmodontis* genomic DNA.

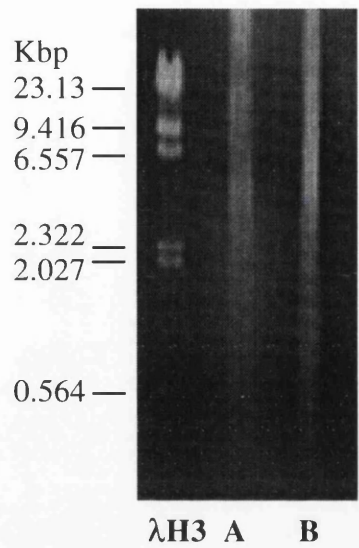


Figure 5.19b *L. sigmodontis* *mmc-1* blot.

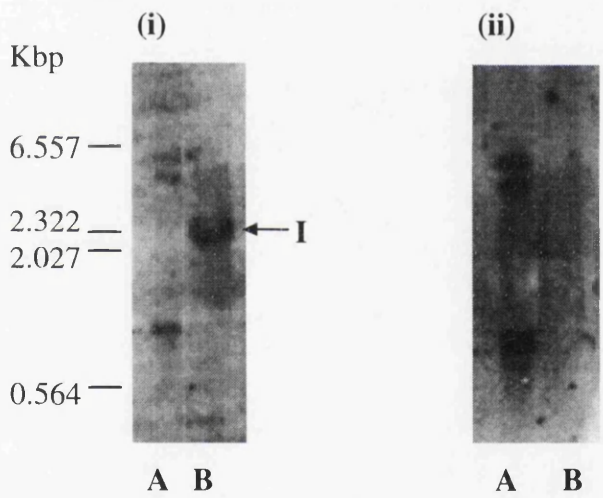


Figure 5.20a.

High molecular weight DNA isolated from *B. pahangi* and *L. loa* filarial worms was digested with *Dde* I, electrophoresed on a 0.8 % agarose gel and visualised under UV light to show approximately equal loading of samples.

λ H3 = *Hind* III digested λ DNA molecular size markers.

A = 15 μ g of *Dde* I digested *B. pahangi* genomic DNA.

B = 15 μ g of *Dde* I digested *L. loa* genomic DNA.

Figure 5.20b(i).

The Southern blot produced from the electrophoresed material shown in Figure 5.14a was hybridised at low stringency with a *mmc-1F1-R1* cDNA probe (50°C, washed 2 X SSC 0.1% SDS 2 x 10 minutes). The autoradiograph was exposed for 72 hours at -70°C. Lanes as shown for Figure 5.20a. The cross reaction seen between the *mmc-1* probe and both *B. pahangi* and *L. loa* DNA at the low stringency wash is marked I.

Figure 5.20b(ii).

Higher stringency wash, the *L. loa* zooblot was washed to slightly higher stringency (50°C, washed 1 X SSC 0.1% SDS 2 x 10 minutes). The autoradiograph was exposed for 72 hours at -70°C. Lanes as shown for Figure 5.20a.

Figure 5.20a Ethidium bromide stained *B. pahangi* and *Loa loa* genomic DNA.

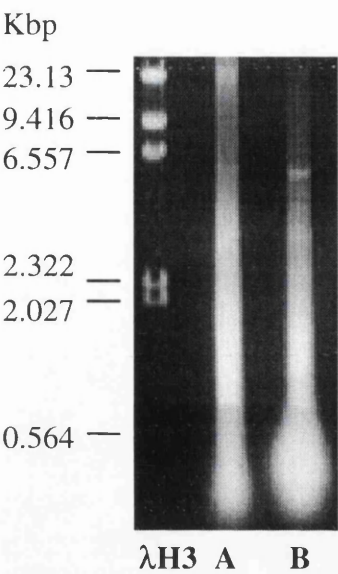
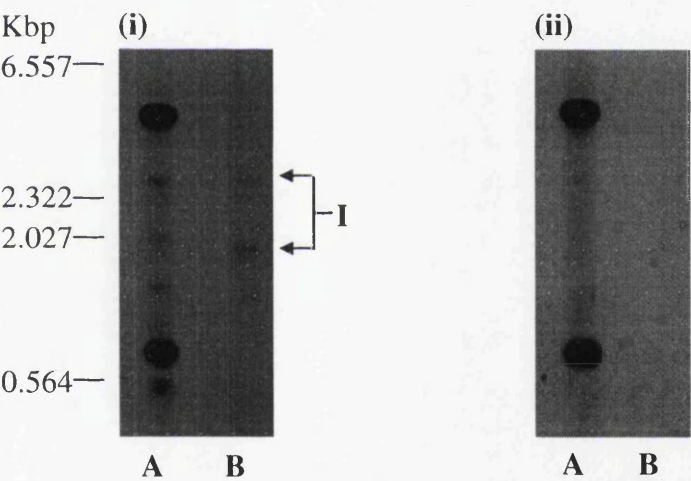


Figure 5.20b *L. loa* *mmc-1* blot.



The final species compared was *W. bancrofti*. This analysis was conducted by PCR on a mf library (SAW95SjL-WbMf Microfilaria conventional cDNA library) provided by the Filarial Genome Project Resource Centre (Smith College Northampton, MA 01063, USA), as genomic DNA from *W. bancrofti* was not available. PCR was conducted at low stringency (50°C annealing temperature) using *mmc-1*F1 and *mmc-1*R1 primers under standard conditions, an aliquot of the *B. pahangi* mammalian-derived mf library was also amplified as a control. The *W. bancrofti* PCR produced no specific bands that could be visualised by EtBr staining (Figure 5.21a). The amplified DNA was transferred to nylon membrane and probed at low stringency with the *mmc-1* cDNA probe. The resulting autoradiograph is shown in Figure 5.21b. As can be seen the *mmc-1* probe does not hybridise to the *W. bancrofti* samples (A and B) or the negative control lane (lane C), but hybridises to a single band of 174 bp from the *B. pahangi* library (marked I). The results show that of the species tested the *mmc-1* probe hybridised only to the *B. pahangi* DNA. Thus *mmc-1* appears to be a *Brugia* specific gene.

5.2.8 Temporal expression of *mmc-1* *in vivo*.

To determine the expression pattern of *mmc-1* mRNA *in vivo* a set of semi-quantitative RT-PCR experiments were conducted as described for *vmc-2* (section 4.2.6). At the outset of the analysis the amplification of *mmc-1* and β -*tubulin* was titrated. 10 μ l of the amplified products after 10, 15, 20, 25, 30 and 35 cycles of PCR were electrophoresed, transferred to nylon membrane and probed with the corresponding gene-specific probe. The degree of amplification was determined by scintillation counting of the specific band (section 2.6). The resulting

Figure 5.21a

Low stringency PCR amplification of *B. pahangi* mammalian-derived mf and *W. bancrofti* mf cDNA libraries with *mmc*-1F1 and *mmc*-1R1 primers. An aliquot of each library was PCR amplified as follows: thirty cycles of: 94°C 1 minute, 50°C 1 minute, 72°C 1 minute. Amplified products were size separated in a 2 % agarose gel. No amplified products could be seen in the *W. bancrofti* cDNA lanes (A and B), or in the negative control lane (C). A single band of predicted size (174bp, marked I) was observed in the control *B. pahangi* cDNA lane, (D).

λH3 = *Hind* III digested λ DNA molecular size markers.

A = 2 µl *W. bancrofti* cDNA library, *mmc*-1F1/R1 primers.

B = 5 µl *W. bancrofti* cDNA library, *mmc*-1F1/R1 primers.

C = No template DNA control PCR, *mmc*-1F1/R1 primers.

D = 2 µl *B. pahangi* cDNA library, *mmc*-1F1/R1 primers.

Figure 5.21b.

The PCR products shown in Figure 5.21a were transferred to a nylon membrane and hybridised with the *B. pahangi mmc*-1F1R1 cDNA probe. The hybridisation and washing was conducted at low stringency (50°C washed 2 x 10 minutes, 2 x SSC 0.1 % SDS). The resulting autoradiograph was exposed to X-ray film for 48 hours at -70°C. Lanes as described above.

Figure 5.21a. PCR amplification of *W. bancrofti* and *B. pahangi* mf libraries with *mmc-1F1*/*mmc-1R1* primers.

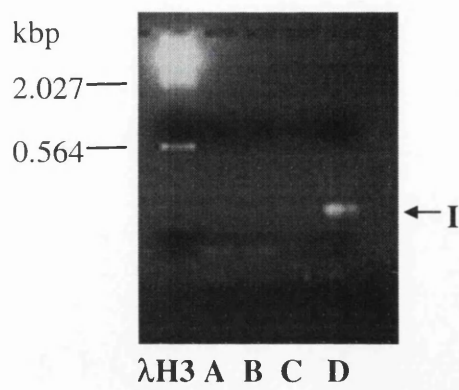
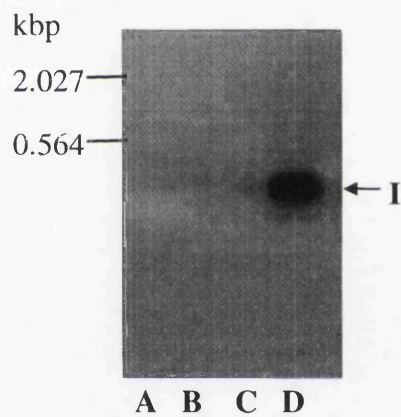


Figure 5.21b. Southern blot of *W. bancrofti* and *B. pahangi* mf library PCR.



autoradiographs are shown in Figure 5.22. From the graphical representation of this data as shown in Figure 5.23, 23 cycles of PCR was chosen as this was in the early exponential stage of amplification for both *mmc-1* and the control gene β -*tubulin*.

The relative abundance of *mmc-1* was determined (as described for *vmc-2* in the previous chapter), by comparison of products amplified by *mmc-1*F1/*mmc-1*R1 primers and those amplified by β -*tubA*/ β -*tubB* primers. Three separate experiments were conducted to investigate the expression of *mmc-1*; Figure 5.24 shows an example of a typical RT-PCR experiment and Table 5.2 shows the counts obtained from the three RT-PCR experiments. Figure 5.25 shows the average of the three experiments in a graphical form. As can be seen from Figure 5.24 *mmc-1* is a gene that is essentially expressed only in the mature mammalian mf stage of *B. pahangi*. No signal was detectable in the infected mosquito stages or from other mammalian derived life-cycle stages. Although no signal was obtained from the mosquito thoraces at 24 hours p.i., mf which were cultured *in vitro* in the vector-like conditions can be seen to express *mmc-1* (Figure 5.24 lane M).

5.2.9 Expression of *mmc-1* in mf *in utero*.

In an attempt to detect low level *mmc-1* expression in mf at different stages of development, adult female worms were dissected and assayed for expression by RT-PCR. Ten gravid females were cut into four approximately equal portions; head, mid I, mid II and tail. The intrauterine development of *Brugia* is synchronous along the length of the uterus. Therefore the head section contained the most mature mf prior to release, which were elongate and free swimming, sections mid I and mid II contained progressively less mature mf and the tail section contained fertilised oocytes and

Figure 5.22 Titration of RT-PCR reactions.

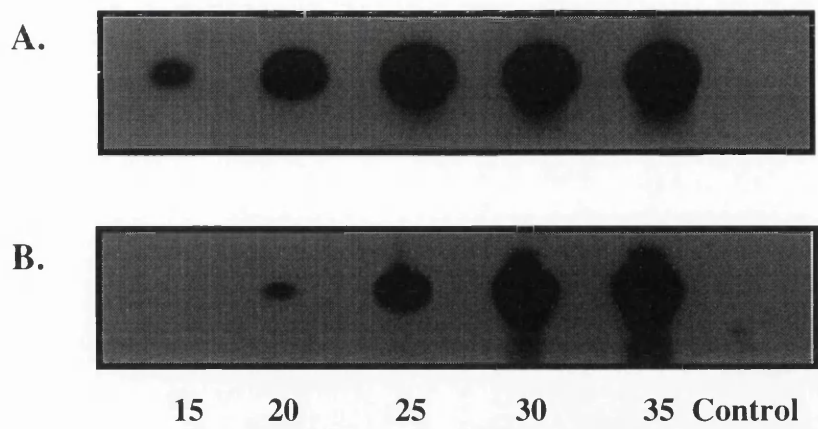


Figure 5.22

Autoradiographs showing titration of PCR amplification using *mmc*-1F1-R1 and β -*tubulin*A-B primer pairs on mammalian-derived mf first strand cDNA. A 100 μ l PCR was conducted under the following conditions, n cycles of: 94°C 1 minute, 55°C 1 minute, 72°C 1 minute. After the number of cycles indicated 12 μ l of each reaction was removed. 10 μ l from each time point was then electrophoresed in a 1% agarose gel. The amplified products were transferred to nylon membrane and probed with the corresponding gene specific probe. The resulting Southern blots were washed at 65°C as follows: 2 x 10 minutes 2x SSC 0.1% SDS, 2 x 10 minutes 1x SSC 0.1% SDS, 2 x 10 minutes 0.1x SSC 0.1% SDS.

A. = *mmc*-1F1-R1 amplified products.
B. = β -*tubulin*A-B amplified products.

Figure 5.23 Titration of RT-PCR reaction.

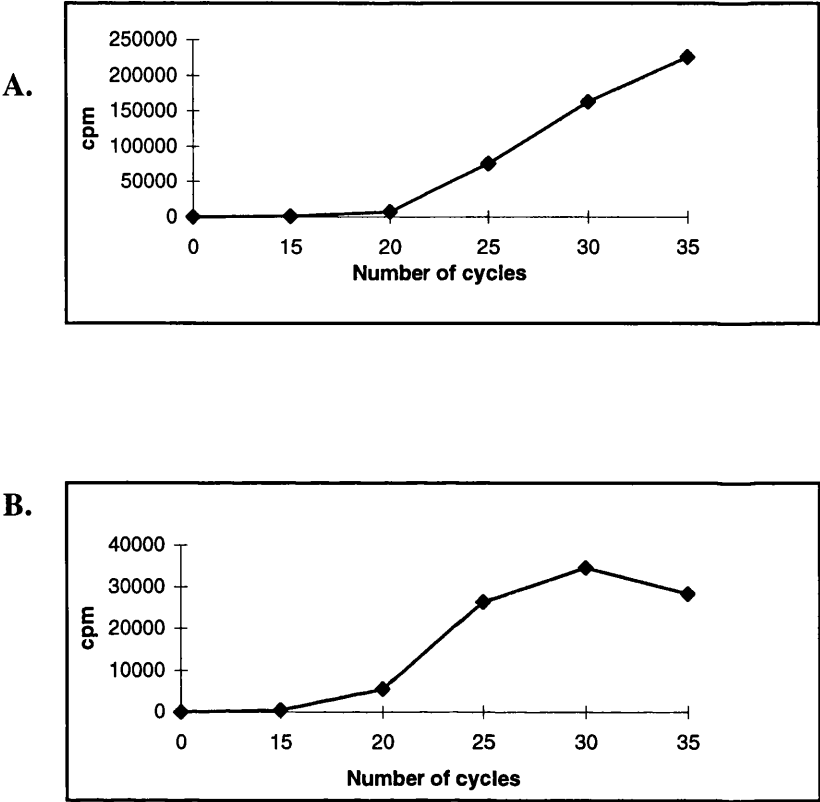


Figure 5.23
Graphical representation of data shown in Figure 5.22 Amplification of PCR products is shown as the incorporated cpm versus the number of cycles. From the graphs 23 cycles was chosen as it is in the initial phase of exponential amplification for both of the primer pairs.

A. = *mmc-1* PCR.
B. = β -*tubulin* PCR

Figure 5.24 *mmc-1* life-cycle panel semi-quantitative RT-PCR.

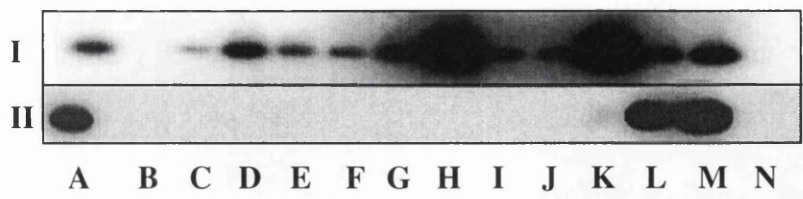


Figure 5.24

First strand cDNA amplified using either β tubA and β tubB primers panel I, or *mmc*-1F1 and *mmc*-1R1 primers panel II, under the following conditions: thirty cycles of: 94°C 1 minute, 62°C 1 minute, 72°C 1 minute. Amplified products were size separated in a 1 % agarose gel and transferred to nylon membrane. The resulting blots were probed with the corresponding gene specific probe under high stringency (65°C, washed to 0.1 x SSC 0.1 % SDS) and exposed to autoradiographic film.

Panel I = β -tubulin RT- PCR.

Panel II = *mmc*-1 RT-PCR.

A = Mature mf.

B = Uninfected mosquito thorax.

C = Mosquito thorax 24 hour post infection (mf).

D = Mosquito thorax 3 days post infection (sausage stage larvae)

E = Mosquito thorax 5 days post infection (L2).

F = Mosquito thorax 8 days post infection (L3).

G = Pre-infective L3 isolated from mosquito into Grace's medium.

H = 24 post infection, mammal (L3).

I = 5 day post infection, mammal (L3).

J = 10 day post infection, mammal (L4).

K = Adults worms.

L = *In vitro* mammalian-derived mf cDNA.

M = *In vitro* vector-derived mf cDNA.

N = No template control.

Table 5.2 *mmc-1* life-cycle RT-PCR scintillation counts.

exp #1	mf	uninfected mosquito	24 hr p.i	3 day p.i	5 day p.i	8 day p.i	Grace's L3	24 hr p.i mammal	5 day p.i	10 day p.i	Adult	vector-derived mf	mammalian-derived mf	n o template DNA control
<i>B-tubulin</i>	812	240	451	2222	663	758	2609	51309	1148	773	70251	1482	4009	291
<i>mmc-1</i>	2203	245	241	262	313	332	749	295	174	244	542	4318	9033	259
<i>B-tubulin</i> minus control	521	-51	160	1931	372	467	2318	51018	857	482	69960	1191	3718	-
<i>mmc-1</i> minus control	1944	-14	-18	3	54	73	490	36	-85	-15	283	4059	8774	-
<i>mmc-1/B-tubulin</i>	3.73	0.27	-0.11	0.00	0.15	0.16	0.21	0.00	-0.10	-0.03	0.00	3.41	2.36	-

exp #2	mf	uninfected mosquito	24 hr p.i	3 day p.i	5 day p.i	8 day p.i	Grace's L3	24 hr p.i mammal	5 day p.i	10 day p.i	Adult	vector-derived mf	mammalian-derived mf	n o template DNA control
<i>B-tubulin</i>	1288	280	464	1360	416	650	3738	22507	1134	638	59476	1638	3447	194
<i>mmc-1</i>	2521	265	221	352	338	377	861	349	239	263	4919	662	9632	280
<i>B-tubulin</i> minus control	1094	86	270	1166	222	456	3544	22313	940	444	59282	1444	3253	-
<i>mmc-1</i> minus control	2241	-15	-59	72	58	97	581	69	-41	-17	4639	382	9352	-
<i>mmc-1/B-tubulin</i>	2.05	-0.17	-0.22	0.06	0.26	0.21	0.16	0.00	-0.04	-0.04	0.08	0.26	2.87	-

exp #3	mf	uninfected mosquito	24 hr p.i	3 day p.i	5 day p.i	8 day p.i	Grace's L3	24 hr p.i mammal	5 day p.i	10 day p.i	Adult	vector-derived mf	mammalian-derived mf	n o template DNA control
<i>B-tubulin</i>	1216	280	403	1285	343	532	3156	19640	925	678	55621	1399	2181	202
<i>mmc-1</i>	2993	269	222	247	274	272	655	284	227	268	489	3337	9109	250
<i>B-tubulin</i> minus control	1014	78	201	1083	141	330	2954	19438	723	476	55419	1197	1979	-
<i>mmc-1</i> minus control	2743	19	-28	-3	24	22	405	34	-23	18	239	3087	8859	-
<i>mmc-1/B-tubulin</i>	2.71	0.24	-0.14	0.00	0.17	0.07	0.14	0.00	-0.03	0.04	0.00	2.58	4.48	-

Mean <i>mmc-1/B-tubulin</i>	2.83	0.11	-0.16	0.02	0.19	0.15	0.17	0.00	-0.06	-0.01	0.03	2.08	3.24
Standard deviations	0.848	0.251	0.055	0.036	0.061	0.074	0.038	0.001	0.036	0.042	0.043	1.629	1.104

Table 5.2

Raw data obtained from triplicate *mmc-1* RT-PCR experiments using cDNAs from throughout the life-cycle. All data is expressed in CPM as determined by scintillation counting of *mmc-1* and β -*tubulin* hybridising cDNA.

Figure 5.25 Relative levels of *mmc-1* expression during *B. pahangi* life-cycle.

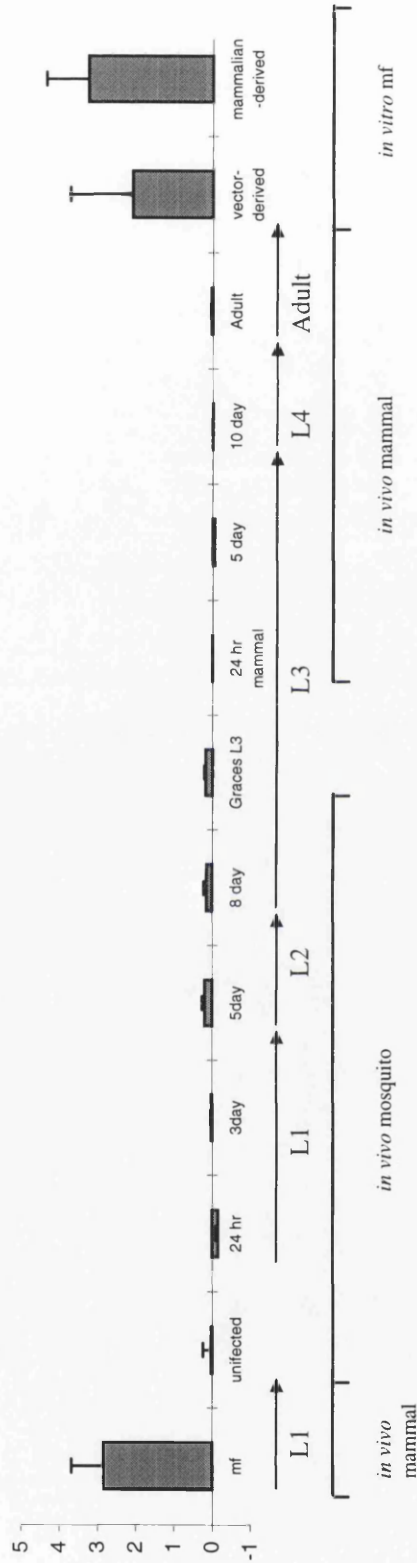


Figure 5.25

The graph shows the relative levels of *mmc-1* expression throughout the life cycle as compared to that of β -tubulin. The data represents the mean values obtained from three semi-quantitative RT-PCR experiments as shown in Table 5.2. The error bars represent the standard deviations from, the mean of the data from the three experiments. The levels of expression of *mmc-1*/ β -tubulin are in arbitrary units. As can be seen *mmc-1* expression is essentially exclusive to the mature mf stage of the life-cycle. The figure also shows the stage of development and the source of the material for production of the staged cDNAs.

oogonia. From each section RNA was isolated and first strand cDNA was synthesised (sections 2.2.15 and 2.2.18 respectively). The expression of *mmc-1* was determined by semi-quantitative RT-PCR by comparison to β -*tubulin* expression as described previously. The *mmc-1* expression from each section was compared to that of first strand cDNA from the following samples: mature mf (>3 months infection of a jird), whole adult males and whole adult females. Figure 5.26 shows an example of one such RT-PCR experiment. The raw data obtained from duplicate experiments is shown in Table 5.3, whilst Figure 5.27 shows a graphical representation of the relative levels of *mmc-1* expression in each of the samples. The graph shows that whilst very low levels of expression are seen in the head and mid I sections the high levels of expression of *mmc-1* are only seen in the mature mf.

5.2.10 Expression of *mmc-1* in mf *in vitro*.

In an attempt to determine at what time point *mmc-1* expression could be detected in the mf of *B. pahangi* mixed sex adult parasites were isolated and incubated in RPMI medium (Gibco) plus supplements (10% FCS, 1% glucose, 2 mM L-glutamine, 2.5 mM HEPES, 100 U/ml penicillin and 100 µg/ml streptomycin). After 6, 18, 24 and 72 hours of culture the medium was removed and the mf collected by centrifugation (1000 rpm, 5 minutes). The mf were used to produce first strand cDNA and the expression of *mmc-1* tested by RT-PCR. Due to the limiting amounts of material the number of PCR cycles was increased to 28 cycles, which was still in the log phase of amplification (see Figure 5.23). The data show that *mmc-1* expression can be detected in mf released from the adult female during six hours in

Figure 5.26 Example of RT-PCR analysis of *mmc-1* expression of mf developing *in utero*.

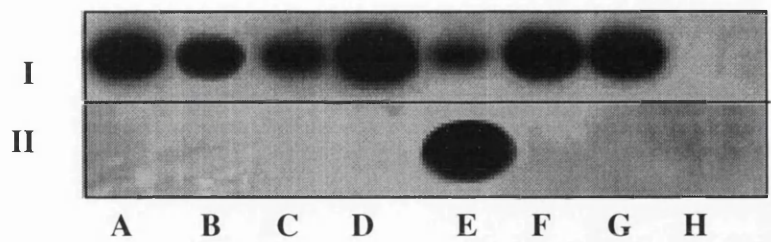


Figure 5.26

An example autoradiograph resulting from RT-PCR expression of mf developing *in utero*. Adult female *B. pahangi* were dissected into four approximately equal sections, head, mid I, mid II and tail. First strand cDNA from each of the sections and from mature mf (> 3 months infection of jird) was amplified using *mmc-1* and β -*tubulin* gene specific primer pairs as follows, twenty eight cycles of: 94°C 1 minute, 62°C 1 minute, 72°C 1 minute. Amplified products were size separated in a 1 % agarose gel and transferred to nylon membrane. The resulting blots were probed with the corresponding gene specific probe under high stringency (65°C, washed to 0.1 x SSC 0.1 % SDS) and exposed to autoradiographic film.

Panel I = β -*tubulin* amplified cDNA

Panel II = *mmc-1* amplified cDNA

A = Adult female section head

B = Adult female section mid I

C = Adult female section mid II

D = Adult female section tail

E = Mature mf

F = Adult female

G = Adult male

H = No template DNA control

Table 5.3 Relative expression of *mmc-1* in sectioned adult females.

Experiment 1	head	mid I	mid II	tail	mature mf	adult male	adult female	control
<i>B-tubulin</i>	484	579	351	415	502	702	619	273
<i>mmc-1</i>	306	325	274	257	802	282	362	328
<i>B-tubulin</i> minus control	211	306	78	142	229	429	346	
<i>mmc-1</i> minus control	-22	-3	-54	-71	474	-46	34	
<i>mmc-1/B-tubulin</i>	-0.104	-0.010	-0.692	-0.5	2.070	-0.107	0.098	

Experiment 2	head	mid I	mid II	tail	mature mf	adult male	adult female	control
<i>B-tubulin</i>	302	358	280	262	580	423	591	380
<i>mmc-1</i>	372	388	396	432	663	380	428	393
<i>B-tubulin</i> minus control	-78	-22	-100	-118	200	43	211	
<i>mmc-1</i> minus control	-21	-5	3	39	270	-13	35	
<i>mmc-1/B-tubulin</i>	0.269	0.227	-0.03	-0.331	1.35	-0.302	0.166	

mean <i>mmc-1/B-tubulin</i>	0.082	0.109	-0.361	-0.415	1.71	-0.205	0.132	
standard deviation	0.264	0.168	0.468	0.12	0.509	0.138	0.048	

Table 5.3
Raw data obtained from duplicate *mmc-1* RT-PCR experiments using cDNAs from sectioned gravid adult females, mature mf, whole adult females and adult males. All data is expressed in CPM as determined by scintillation counting of *mmc-1* and β -*tubulin* hybridising cDNA.

Figure 5.27 Relative levels of *mmc-1* expression in mf stages *in utero*.

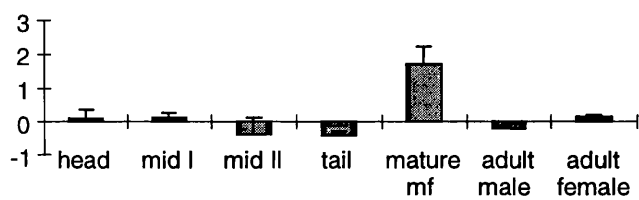


Figure 5.27
The graph shows the relative levels of *mmc-1* expression of mf developing *in utero* as compared to that of β -*tubulin*. Gravid adult female worms were dissected into four approximately equal sections, head, mid I, mid II and tail. The level of expression in the sectioned females is compared to that of mature mf (> 3 months) and whole adult male and female worms. The data represents the mean values obtained from duplicate semi-quantitative RT-PCR experiments as shown in Table 5.3. The error bars represent the standard deviations from, the mean of the data from the experiments. The levels of expression of *mmc-1*/ β -*tubulin* are in arbitrary units.

culture (Figure 5.28). Due to the limitations imposed by the low numbers of mf released it was not possible to conduct this type of experiment on earlier time points.

As *mmc-1* expression is detectable within hours of release of the mf from the adult female *in vitro*, attempts were made to manipulate the culture medium to determine the factors that influence expression. The temperature of culture and the presence of serum and glucose concentrations were tested. For this set of experiments the input first strand cDNA was normalised by comparison to standards of known concentration on EtBr plates (section 2.6.1). RT-PCR was then conducted using the *mmc-1* primer pair as before. The level of expression was then directly compared by EtBr staining. In this way the data obtained was not quantifiable but was comparable between groups. RT-PCR of the cDNAs using the β -*tubulin* primer pairs was also conducted but only as an internal control of cDNA quality, as no comparisons to the levels of *mmc-1* expression were carried out. Using this procedure any variation of β -*tubulin* gene expression as a result of the culture conditions would not affect the interpretation of *mmc-1* expression.

5.2.10.1 Does temperature influence *mmc-1* expression?

Approximately 1×10^6 mf isolated from the peritoneal cavity of an infected jird were purified from contaminating mammalian cells (section 2.1.4) and incubated for 2 hours at either 28°C, 37°C or 41°C in RPMI + 10 % FCS. RNA was extracted from the worms and first strand cDNA synthesis conducted under standard conditions. The cDNA was quantified using EtBr staining and then normalised to approximately 5 ng/ μ l (see Figure 5.29 panel III). cDNA produced from *ex vivo* mf directly purified from the peritoneal cavity of the jird was also analysed. The cDNA

Figure 5.28 Early expression of *mmc-1* *in vitro*.

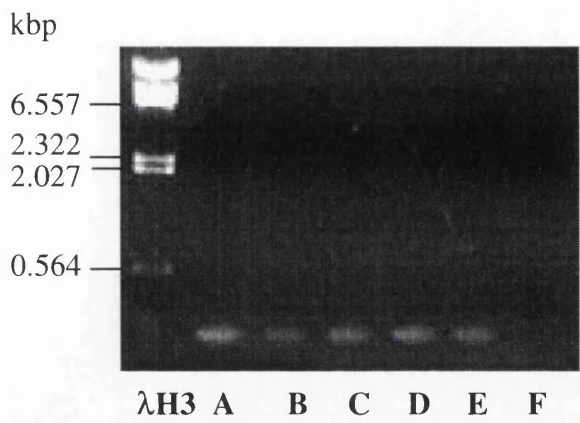


Figure 5.28

Mf released from cultured adult females were collected after 6, 18, 24 and 72 hours. First strand cDNA was amplified using *mmc-1* and β -*tubulin* gene specific primer pairs as follows, twenty eight cycles of: 94°C 1 minute, 62°C 1 minute, 72°C 1 minute. Amplified products were size separated in a 1 % agarose gel and transferred to nylon membrane. The resulting blots were probed with the corresponding gene specific probe under high stringency (65°C, washed to 0.1 x SSC 0.1 % SDS) and exposed to autoradiographic film.

λH3 = *Hind* III digested lambda DNA size markers.

A = 6 hour culture

B = 18 hour culture

C = 24 hour culture

D = 72 hour culture

E = Mature *ex vivo* mf

F = No template DNA control

Figure 5.29 Expression of *mmc-1* in mf cultured at 28°C, 37°C and 41°C.

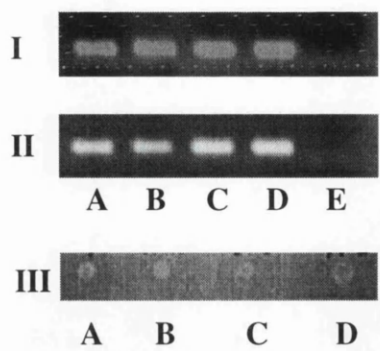


Figure 5.29

Levels of *mmc-1* in RT-PCR amplified cDNA from mf cultured for 2 hours at 28°C, 37°C 41°C and mature *ex vivo* mature mf (> 3 months). Normalised first strand cDNA was amplified using *mmc-1* and β -*tubulin* gene specific primer pairs as follows, twenty eight cycles of: 94°C 1 minute, 62°C 1 minute, 72°C 1 minute. Amplified products were size separated in a 1 % agarose gel and transferred to nylon membrane. The resulting blots were probed with the corresponding gene specific probe under high stringency (65°C, washed to 0.1 x SSC 0.1 % SDS) and exposed to autoradiographic film.

Panel I = *mmc-1* amplified cDNA

Panel II = β -*tubulin* amplified cDNA

Panel III = 0.5 μ l normalised cDNA samples compared by EtBr staining

A = Mature mf

B = 28°C cultured mf

C = 37°C cultured mf

D = 41°C cultured mf

E = No template DNA control

from each group was then amplified for 28 cycles using the *mmc*-1F1 and *mmc*-1R1 primer pair and the β -*tubulin*A and β -*tubulin* B primer. The amplified products were separated on a 2% agarose gel, stained with EtBr and visualised by UV illumination. No differences in the levels of expression could be detected between the groups in this or in replicate experiments (Figure 5.29).

5.2.10.2 Does the presence of FCS or glucose affect the expression of *mmc*-1?

In an attempt to further dissect factors that may be influencing the expression of *mmc*-1 *in vitro*, a number of culture experiments were conducted in which constituents of the medium were altered. Mixed sex adult parasites were incubated for 24 hours in 25 cm² culture flasks (as described in section 2.8) and expression of *mmc*-1 by released mf was determined by RT-PCR analysis on normalised cDNA samples. Two of the major factors that were possible candidates for manipulation were presence of FCS and glucose. Initially adult parasites were incubated for 24 hours in RPMI medium \pm 10 % FCS, \pm 1 % glucose and the released mf collected as described in section 5.2.10. RNA was isolated and first strand synthesis was conducted by standard means, the levels of expression were compared by RT-PCR to that of *ex vivo* derived mf. The results of duplicate culture experiments showed that the absence of either FCS or glucose had no effect on the *mmc*-1 expression as determined by EtBr staining (Figure 5.30).

The RPMI used in the above experiments has a glucose concentration of 2g/L. It was therefore possible that this was sufficient to stimulate the expression of *mmc*-1 *in vitro*. In order to determine if the total absence of glucose would influence the expression of *mmc*-1, RPMI medium with no glucose (Gibco Life Technologies

Figure 5.30 Expression of *mmc-1* in mf cultured in RPMI medium \pm 10 % FCS and \pm 1 % glucose

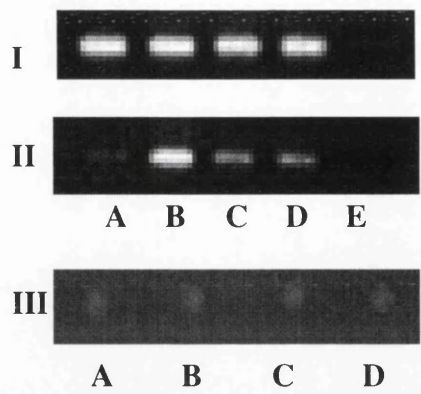


Figure 5.30
Levels of *mmc-1* in RT-PCR amplified cDNA from mf cultured for 24 hours in RPMI medium \pm 10 % FCS and \pm 1 glucose at 37°C. Normalised first strand cDNA was amplified using *mmc-1* and β -*tubulin* gene specific primer pairs as follows, twenty eight cycles of: 94°C 1 minute, 62°C 1 minute, 72°C 1 minute. Amplified products were size separated in a 1 % agarose gel and transferred to nylon membrane. The resulting blots were probed with the corresponding gene specific probe under high stringency (65°C, washed to 0.1 x SSC 0.1 % SDS) and exposed to autoradiographic film.

- Panel I = *mmc-1* amplified cDNA
- Panel II = β -*tubulin* amplified cDNA
- Panel III = 0.5 μ l normalised cDNA samples compared by EtBr staining
- A = Mature *ex vivo* mf
- B = RPMI + 10 % FCS + 1 % glucose
- C = RPMI + 10 % FCS - 1 % glucose
- D = RPMI - 10 % FCS + 1 % glucose
- E = No template DNA control

catalogue number 51872-018, RPMI-g) was used to culture adult parasites and the level of *mmc-1* expression in the released mf determined as before. Prior to the culture experiments the concentration of glucose in each of the media was tested (Table 5.4). Adult parasites were incubated for 24 hours and the mf were collected, then the medium was replaced and a further 24 hour incubation was conducted. This was again repeated and the adults incubated for a further 72 hours. Again when RT-PCR of the resultant cDNA was conducted no visible differences were detected between any of the groups (Figure 5.31).

The numbers of mf released by the females in each of the experiments was also recorded. No significant differences in mf numbers were seen between the media tested after 24 hour incubation (data not shown). The slight discrepancies in mf numbers were likely to be due to the counting procedure where the number of mf in two 20 µl aliquots of media was counted and the total per ml calculated. However, differences were seen between the RPMI and RPMI-g media. After the first 24 hour period both the cultures produced approximately equal numbers of mf. After the second 24 hour incubation the release of the mf from the females in the RPMI media remained steady, whilst the numbers of mf were reduced in the no-glucose media. This trend was more evident during the 72 hour culture period (Figure 5.32).

5.3 Attempts to isolate the upstream region of *mmc-1*.

5.3.1 Screening of a *Brugia pahangi* genomic DNA library.

As the experiments described above were unsuccessful in identifying possible triggers for *mmc-1* expression a different approach was adopted, in order to clone and sequence the upstream region of *mmc-1*. By these methods it may be possible to

Table 5.4 Glucose concentrations of culture media.

Medium	Glucose concentration (mM)
RPMI (Dutch modification)	10.2
RPMI (Dutch modification) + 10 % FCS	10.1
RPMI (Dutch modification) + 1 % glucose	69.3
RPMI (Dutch modification) + 10 % FCS + 1 % glucose	68.0
RPMI (No glucose)	0.0
RPMI (No glucose) + 10 FCS	0.8

Table 5.4
Glucose concentrations of various culture media as determined by the Hexokinase method (Bayer Plc, Berkshire).

Figure 5.31 Expression of *mmc-1* in mf cultured in RPMI medium (No glucose) + 10 % FCS after 24 and 72 hour cultures.

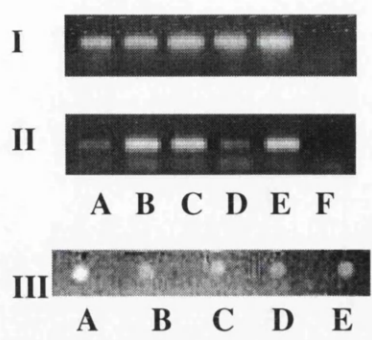


Figure 5.31
levels of *mmc-1* in RT-PCR amplified cDNA from mf cultured for 24 or 72 hours in RPMI medium (no glucose RPMI-g) + 10 % FCS at 37°C. Normalised first strand cDNA was amplified using *mmc-1* and β -tubulin gene specific primer pairs as follows, twenty eight cycles of: 94°C 1 minute, 62°C 1 minute, 72°C 1 minute. Amplified products were size separated in a 1 % agarose gel and transferred to nylon membrane. The resulting blots were probed with the corresponding gene specific probe under high stringency (65°C, washed to 0.1 x SSC 0.1 % SDS) and exposed to autoradiographic film.

Panel I = *mmc-1* amplified cDNA
Panel II = β -tubulin amplified cDNA
Panel III = 0.5 μ l normalised cDNA samples compared by EtBr staining
A = Mature *ex vivo* mf
B = 24 hours RPMI + 10 % FCS + 1 % glucose
C = 24 hours RPMI-g + 10 % FCS
D = 72 hours RPMI 10 % FCS + 1 % glucose
E = 72 hours RPMI-g 10 % FCS
F = No template DNA control

Figure 5.32 Mf production of adult females cultured in RPMI versus RPMI-g

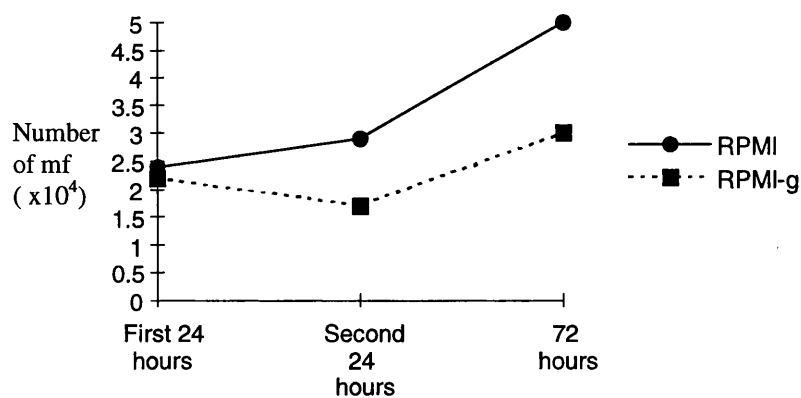


Figure 5.32
Graph of the number of mf released by cultured adult females after consecutive 24 and 72 hour cultures. Adults cultured at 37°C in either RPMI + 10 % FCS + 1 % glucose or RPMI-g + 10 % FCS.

identify possible regulatory elements in the 5' region of the gene. After PCR using *mmc-1F1* and *mmc-1R1* primers to confirm the presence of *mmc-1* in the library a screen of a *B. pahangi* genomic DNA library was attempted. 1×10^6 pfu of a *B. pahangi* genomic DNA library in λ DASH II (provided by Dr Fiona Thompson, University of Glasgow) was screened by hybridisation with a *mmc-1* genomic DNA probe (see section 2.2.22.1). The primary screen (55°C, washed to $1 \times$ SSC, 0.1 % SDS) identified 7 positively hybridising plaques, which were subjected to a secondary screen as before and 24 positive clones were isolated. Five randomly picked secondary screen positive plaques were subjected to tertiary screening at higher stringency (60°C, washed to $0.1 \times$ SSC, 0.1 % SDS). The tertiary screen did not produce any positive hybridisation. The primary and secondary screen positives were assessed for the presence of a *mmc-1* insert by PCR with gene specific primers (*mmc-1F1/mmc-1R1*), but no positive amplification was seen (results not shown). This confirmed the suspicion that the hybridisation seen in the primary and secondary rounds were false positives.

5.3.2 Cloning *mmc-1* upstream region by PCR.

To obtain the upstream region without the need for hybridisation to total genomic DNA, a PCR technique was employed. It was hoped that by long-range PCR amplification of the *B. pahangi* genomic library [205, 206], using gene specific primers in conjunction with the lambda primers T3 and T7 the up-stream region could be isolated without the need for extensive subcloning. A schematic of the positions of primers used in the long range PCR techniques is shown in Figure 5.33. It has been shown that A+T rich templates may be problematic to amplify using

Figure 5.33 Positions of primers used in long-range PCR.

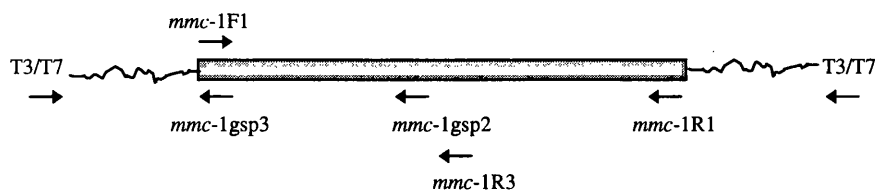


Figure 5.33
Schematic diagram showing the positions of primers used in the long-range PCR techniques to clone the upstream region of *mmc-1*. The shaded box represents the *mmc-1F1/mmc-1R1* genomic fragment within a typical λ clone from the library.

standard PCR protocols, due to the melting of the polymerase and template at the standard extension temperature of 72°C [164]. This was considered a potential problem when amplifying the *mmc-1* genomic sequence which has particularly A+T rich introns (90 % A+T intron 1, 79 % A+T intron 2). The PCRs were therefore conducted using a more robust DNA polymerase (AGSGold, Hybaid), and modified conditions (50°C annealing, 60°C extension, see section 2.2.22.2) as suggested by Su *et al* (1996) [164]. The primers *mmc-1R1* and *mmc-1R3* were used in conjunction with either T3 or T7 vector primers. Following thirty cycles of PCR a number of bands were observed in the *mmc-1R1*,T7 and *mmc-1R3*, T7 samples (Figure 5.34 lanes A and C respectively). The origin of the smear of DNA seen in some lanes (E, G, I and K) is not known but reports of such artefacts are a feature of the long range PCR technique [164]. It is likely that the low temperature conditions used in the PCR has resulted in some spurious priming of unrelated sequences; this is evident in the positive control lane (lane M) where the amplification of a *mmc-1F1-mmc-1R3* plasmid resulted in a large number of products, in addition to the predicted 426 bp product (Figure 5.34 marked I). The amplified products were transferred to nylon membrane and probed with a *mmc-1F1-mmc-1R3* genomic DNA probe (Figure 5.35). The Southern blot shows a number of weakly hybridising bands in the *mmc-1R1*/T7 sample (lane A) and two bands of intense hybridisation in the *mmc-1R3*/T7 sample (lane C). The band marked i is of the correct predicted size (996 bp) for the *mmc-1F1/mmc-1R1* control PCR. The band marked ii is of the correct predicted size (436 bp) for the *mmc-1F1/mmc-1R3* control PCR. The two strongly hybridising bands from the *mmc-1R3*/T7 PCR were gel purified and cloned into a TA vector (Invitrogen). The resulting purified plasmid from these transformations were

Figure 5.34 Long-range PCR of *B. pahangi* genomic DNA λ DASH II library to isolate the upstream region of *mmc-1*.

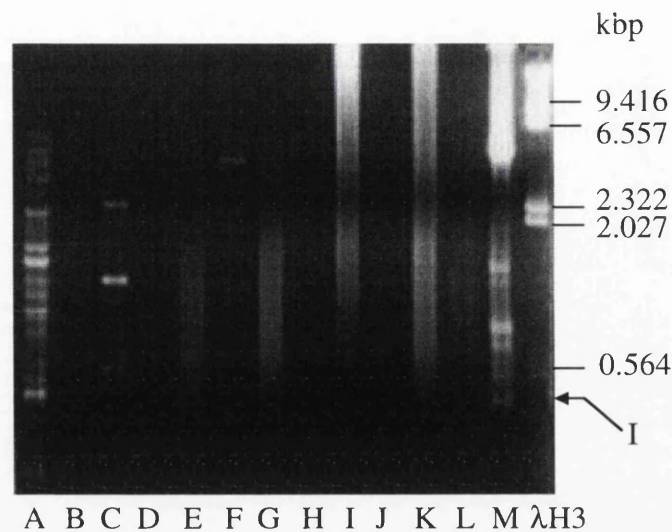


Figure 5.34

An aliquot of a *B. pahangi* genomic DNA library in λ DASH II was boiled for 5 minutes then placed on ice. A 5 μ l aliquot of this was amplified by PCR with AGSGold polymerase using *mmc-1R1* and *mmc-1R3* gene specific primers, and T3 and T7 vector specific primers, under the following conditions: thirty cycles of 94°C 1 minute, 55°C 1 minute, 60°C 10 minutes. 10 μ l of the amplified products were run on a 1 % agarose gel and stained with Ethidium Bromide.

- A = *mmc-1R1*, T7 primers.
- B = *mmc-1R1*, T3 primers.
- C = *mmc-1R3*, T7 primers.
- D = *mmc-1R3*, T3 primers.
- E = *mmc-1F1*, *mmc-1R1* primers.
- F = *mmc-1F1*, *mmc-1R3* primers.
- G = *mmc-1F1*, *mmc-1R1* primers no template DNA control.
- H = *mmc-1F1*, *mmc-1R3* primers no template DNA control.
- I = *mmc-1R1*, T7 primers no template DNA control.
- J = *mmc-1R1*, T3 primers no template DNA control.
- K = *mmc-1R3*, T7 primers no template DNA control.
- L = *mmc-1R3*, T3 primers no template DNA control.
- M = *mmc-1F1-R3* plasmid positive control.
- λH3 = *Hind* III digested λ DNA molecular size markers.

Figure 5.35 Long-range PCR southern blot.

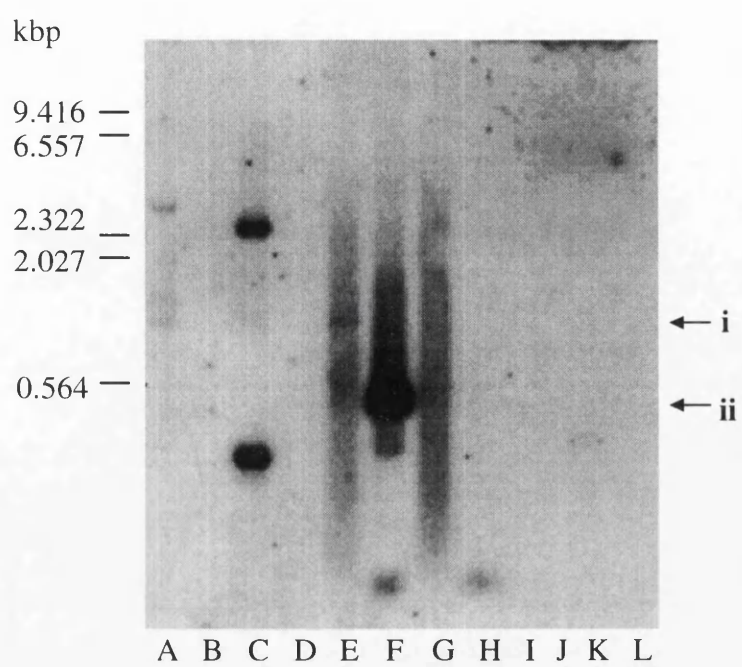


Figure 5.35

Southern blot produced from the electrophoresed material shown in Figure 6.8 was hybridised with a *mmc-1F1-R3* genomic DNA probe (50°C, washed 1 x SSC 0.1% SDS 2 x 10 minutes). The autoradiograph was exposed for 10 days -70°C. Lanes as shown for 5.34. The *mmc-1F1/mmc-1R3* plasmid positive control PCR (lane M Figure 5.34) was removed as the specific activity was very intense and would have led to quenching of the X-ray film. Lanes A-L as described in Figure 5.34. The band marked i is of predicted size (996 bp) for the *mmc-1F1/mmc-1R1* control PCR. The band marked ii is of the predicted size (436 bp) for the *mmc-1F1/mmc-1R3* control PCR.

sequenced on both strands by standard techniques (2.2.20). From the preliminary sequencing it was apparent that the amplified bands were due to false amplification as the *mmc-1* specific primer was present at both ends of the amplified PCR fragment but neither end progressed through the known *mmc-1* DNA into the upstream region. The low stringency of the PCR was likely to have led to the promiscuous annealing of the primer and the results seen.

To increase the chance of specifically amplifying the *mmc-1* upstream region a nested approach was then adopted. PCR was conducted using the same *B. pahangi* genomic library and vector primers as before, but utilising the nested primers *mmc-1R1*, *mmc-1gsp2* and *mmc-1gsp3* (*mmc-1gsp2* and *mmc-1gsp3* used previously for 5' RACE, see section 5.2.3). 5 µl of a 1/10 dilution of the genomic library was amplified for thirty cycles using T3 or T7 primers and *mmc-1R1* (section 2.2.22.2). 1 µl of the round 1 reaction was removed and amplified using *mmc-1gsp2* and the corresponding vector primer. This was again repeated using *mmc-1gsp3* and the vector primers. It was hoped that by the use of nested primers preferential amplification of the *mmc-1* upstream region over any false product would result. The results of the nested PCR are shown in Figure 5.36. The first round PCR (*mmc-1R1* and vector primers) did not produce any visible amplified products, only the control lane of genomic DNA amplified with *mmc-1F1* and *mmc-1R3* produced a band of predicted size (436 bp, Figure 5.36 lane G). The second round of PCR only produced amplified products from the control *mmc-1F1* and *mmc-1R3* amplified lanes (Figure 5.36 lanes I, K and L). The final round of PCR also failed to amplify products using the vector and gene specific primers. The fact that the control reactions I and K produced bands of predicted size shows that *mmc-1* can be amplified and suggests

Figure 5.36 Long-range nested PCR of *B. pahangi* genomic DNA λ DASH II library to isolate the upstream region of *mmc-1*.

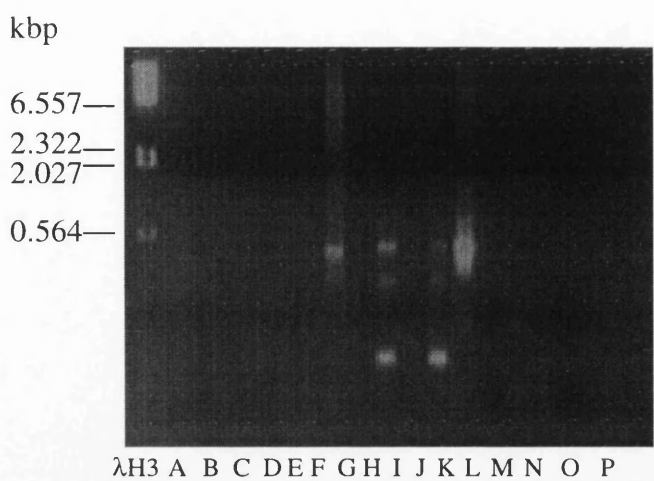


Figure 5.36

An aliquot of a *B. pahangi* genomic DNA library in λ DASH II was boiled for 5 minutes then placed on ice. 5 μ l of a 1/10 dilution of this was amplified by PCR with AGSGold polymerase using nested *mmc-1* gene specific primers, and T3 and T7 vector specific primers, under the following conditions: thirty cycles of 94°C 1 minute, 55°C 1 minute, 60°C 10 minutes. 10 μ l of the amplified products were run on a 2 % agarose gel and stained with Ethidium Bromide.

Round one.

A = *mmc-1*R1, T3 primers.

B = *mmc-1*R1, T7 primers.

C = *mmc-1*F1, *mmc-1* R3 primers.

D = *mmc-1*R1, T3 primers no template DNA control.

E = *mmc-1*R1, T7 primers no template DNA control.

F = *mmc-1*F1, *mmc-1*R3 primers no template DNA control.

G = *mmc-1*F1, *mmc-1*R3 primers 100ng *B. pahangi* genomic DNA positive control.

Round two.

H = 1 μ l of reaction A, *mmc-1*gsp2, T3 primers.

I = 1 μ l of reaction A, *mmc-1*F1, *mmc-1* R3 primers.

J = 1 μ l of reaction B, *mmc-1*gsp2, T7 primers.

K = 1 μ l of reaction B, *mmc-1*F1, *mmc-1* R3 primers.

L = 1 μ l of reaction G, *mmc-1*F1, *mmc-1*R3 primers.

Round three

M = 1 μ l of reaction H, *mmc-1*gsp3, T3 primers.

N = 1 μ l of 1:10 dilution of reaction H, *mmc-1*gsp3, T3 primers.

O = 1 μ l of reaction J, *mmc-1*gsp3, T7 primers.

P = 1 μ l of 1:10 dilution of reaction H, *mmc-1*gsp3, T7 primers.

λH3 = *Hind* III digested λ DNA molecular size markers.

that *mmc-1* is present in the library. However no products were seen after the first round PCR of 30 cycles, but only after re-amplification for a further 30 cycles suggesting that *mmc-1* is under-represented in the library.

5.4 Discussion.

Analysis of the *mmc-1* cDNA produced a number of interesting findings: the ORF encodes a small peptide consisting of 81 amino acids that has a predicted cleavable signal sequence (amino acids 1-17). The *mmc-1* gene product is therefore potentially a secreted protein, and as such has the opportunity to interact with the host environment. As the homologues to *mmc-1* were to cDNAs of unknown function, attempts were made to identify domains in the protein sequence that may be homologous to proteins of known function [207]. The analysis demonstrated a low degree of homology between *mmc-1* and a family of transcription factors. Unfortunately the lack of the signature domain that allows the binding of these transcription factors to DNA meant that consideration of *mmc-1* as a homologous helix-loop-helix containing transcription factor had to be dismissed. The lack of other signatures in the *mmc-1* sequence confirmed that at present *mmc-1* must be regarded as a novel gene without predicted function.

Assigning functions to parasite-specific genes which have no homologues is an increasing problem as more sequences are deposited in parasite EST databases. An increasing amount of research will have to be carried out *in silico* to determine those genes that are of primary importance (for review see [126]). Recent comparison of the *B. malayi* ESTs by comparison with other public databases showed that 38 % are unique to *Brugia* with no known homologues and only 45 % of the ESTs are

homologues of genes of known function [132, 139]. As discussed in the Introduction this may prove to be problematic to parasite biologists as the parasite specific genes are likely to be of most interest.

As the *mmc-1* sequence obtained from the mammalian-derived mf library contained the SL-1 sequence, it was considered unlikely that the *B. malayi* homologue would not. The relevance of the SL1 sequence is unknown, certainly if SL1 sequences are experimentally *trans*-spliced onto mRNAs that do not normally possess SL1, subsequent expression is unaffected [110]. Analysis of the mRNAs known to possess the SL1 sequence shows that there is no common group that can be applied to mRNAs that are *trans*-spliced or those that are not [208]. In the actin group originally found to possess the SL1 sequence one of the four actin mRNAs did not show the sequence [179]. It has also been reported that the mRNAs that are SL *trans*-spliced in one species may not be present in the homologous gene of another [208]. However these studies relate to the comparison of *C. elegans* and schistosome species and the distant relationship of these animals (one a nematode and one a platyhelminth) may explain these differences. It has been proposed that *trans*-splicing of a mRNA molecule removes any extended 5' UTR that may be present thereby promoting accurate translation [110, 208]. In support of this proposal, the resulting 5' UTR of nematodes are typically very short with < 20 bases between the SL1 sequence and the methionine initiation codon [122, 209]. This is seen in *mmc-1* where there is only eight bases between the SL1 sequence and the initiation codon, ATG. While studies carried out in this chapter showed that *Bp-mmc-1* was *trans*-spliced to SL1, the corresponding *B. malayi* ESTs isolated from a conventional cDNA library were not reported to be *trans*-spliced. It was thought unlikely that the

mRNA would be differently *trans*-spliced in *B. malayi* and *B. pahangi*. In conclusion the differences in the *B. malayi* and *B. pahangi* transcripts is likely to be due to the cloning and sequencing procedure used to generate the *B. malayi* ESTs.

The analysis of the northern blot showed that the *mmc-1* transcript is up-regulated in the mammalian-derived mf as was predicted by the differential library screen. The northern blot also demonstrates a low level of expression of *mmc-1* in the vector-derived mf RNA. This suggests that the culture of the parasites in Grace's insect medium does not lead to the complete suppression of *mmc-1* expression. It was necessary to use an *in vitro* system for this analysis as it is impossible to obtain sufficient parasite material from mosquitoes for equivalent analysis.

The RT-PCR analysis carried out confirmed the results of the northern blots and showed the same discrepancy between *mmc-1* expression in mosquito-derived mf *in vivo* (24 hour p.i mosquito stage) and in the vector-derived *in vitro* system, see Table 5.2. The RT-PCR experiments suggested that *mmc-1* was not expressed in the vector-derived mf stage *in vivo* but expression was seen in the parasites cultured *in vitro* (vector-derived = 2.08 ± 1.63). The reasons for this discrepancy are not clear. The most likely explanation is that the culture system used does not properly mimic the conditions to which mf are exposed *in vivo* in the mosquito host. Alternatively the results of the *in vivo* vector-derived RT-PCR may not be as accurate as at other time points. This may be due to the fact that in the mosquito the number of parasites that are present in any one mosquito thorax is limited (approximately 6-20 in the conditions used here) and that size of the L1 parasites is approximately $190 \times 8 \mu\text{m}$ after 24 hours in the mosquito [9]. Therefore parasite mRNA molecules will be

vastly out-numbered by mosquito mRNAs and it may be difficult to detect parasite transcripts which are not abundant by this method.

Comparison of the cDNA and genomic DNA nucleotide sequences shows that *mmc-1* contains two introns and three possible exons, although the possibility of further introns being present outwith the compared *mmc-1F1-mmc-1R1* genomic PCR fragment and the end of the transcript cannot be ruled out. In comparison to a survey of *B. malayi* introns [128] the introns of *mmc-1* are relatively large (338 and 483 bp) although neither is as large as the *vmc-2* intron (716 bp) described in the last chapter, and have a higher percentage A+T than the *B. malayi* consensus. The only complete exon, i.e. flanked by introns is exon 2. This short exon (62 bp) follows the trend seen in *B. malayi* genes where the highest frequency of exon sizes analysed is in the size class 61-80bp [128]. Analysis of the splice site junctions of *B. malayi* revealed a consensus sequence of AG/GT and AG/G for the 5' and 3' splice sites respectively [198]. The *mmc-1* intron boundaries fit this general consensus although the exon sequences are not as strict (CAG/GTA..TAG/AAA intron 1, TGT/GTA..CAG/GCT intron 2). However it is not possible to draw any conclusions re-intron structure in *B. pahangi* on the basis of the analysis carried out here.

As *mmc-1* was clearly only expressed in the mf stage, its function may relate to some aspect of the biology of the parasite which is exclusive to the mf. One feature which is specific to some mf is the presence of a sheath. The fact that *mmc-1* is not expressed by adult females would suggest it is not a structural component of the sheath. Many of the characterised structural components of the sheath (the modified egg shell) are synthesised in the adult female by both the embryo and the uterine epithelium in the adult female [81]. For example the known sheath matrix

proteins such as SHP2 are synthesised during development in the embryo [89]. However this does not preclude *mmc-1* from being associated with the sheath once it is formed, or from having a role associated with the presence of the sheath. An example of such a gene is the microfilarial chitinase of *B. malayi* (MF1, accession number A38221, [210]) the expression of which correlates with the possession of the sheath as it is not expressed in non-sheathed species such as *D. immitis* [211, 212]. The role of the chitinases in the development of *Brugia* mf is not fully understood. Their expression is very low in intrauterine and immature mf but increases as the mf mature [212]. As the fully-developed sheath does not contain detectable amounts of chitin [81, 84], it is unlikely that the chitinases are involved in the process of exsheathment but may instead be involved in the penetration of chitinous structures in the mosquito during the migration of the parasite in the vector [210, 212]. An alternative role relates to the observation that chitin is present in the eggshell of oocytes and zygotes of filarial parasites but not in the mature microfilarial sheath as determined by competitive lectin binding studies [85]. Fuhrham and Piessens (1985) suggested that chitinase activity seen in the early development of the mf may modify the sheath to allow the morphogenesis of the sheath and the unrestricted growth of the mf [83]. Support for this hypothesis comes from experiments in which the inhibition of chitin synthesis was shown to produce folded mf within a truncated sheath [83]. However the analysis carried out here failed to detect *mmc-1* in any of the other filarial parasites examined, either blood dwelling species (*W. bancrofti*, *L. loa*, *A. viteae*, *D. immitis* or *L. sigmodontis*) or in the skin dwelling mf of *O. gibsoni*. Likewise expression of *mmc-1* was not correlated with the possession of a mf sheath as no signal was obtained in *W. bancrofti*, *L. loa* and *L. sigmodontis* DNA compared

to those that are unsheathed (*O. gibsoni*, *A. viteae* and *D. immitis*). Thus it appears that *mmc-1* is a *Brugia* specific gene.

Southern blot analysis of *mmc-1* at very low stringency did show some cross-hybridisation suggesting that *mmc-1* has some sequence homology to another gene or genes in the *B. pahangi* genome. This may also explain the degree of cross-hybridisation seen in the zooblot analysis of *L. sigmodontis* and *L. loa* DNA. The failure to detect a homologue in the *W. bancrofti* library may be a reflection of the experimental procedure. Screening the *W. bancrofti* cDNA library at low stringency may have isolated a more distantly related homologue than the PCR conditions allowed, although this was not possible due to time restrictions.

The uterus of the adult female of *Brugia* is composed of two parallel genital tubes in which development of the embryo progresses from distal to proximal ends [213]. At the distal end of the uterus, lies the ovary in which germ cell formation and maturation takes place [214]. The ovary is located in the hind body of *B. pahangi* but does not extend behind the level of the anus [7]. The oocytes mature in growth zones in the ovary at the proximal end the ovary narrows producing a seminal receptacle, where the oocytes are fertilised and pass into the uterus [214]. Development is synchronous throughout the uterus and as the embryo develops it can be seen to become compact then form a coiled and finally elongate mf [213-215]. The mf are expelled from the adult female at the *vagina vera*, a muscular pyriform ovejector, located 0.49-0.58 mm behind the head [7]. Due to this synchronous development of *Brugia in utero* the expression of *mmc-1* at various stages of maturation could be assessed. RT-PCR analysis of sectioned adult females and the use of *in vitro* culture

techniques allowed the determination of the temporal expression of *mmc-1* at early time points.

The RT-PCR analysis of the sectioned adult females showed that *mmc-1* expression either follows or is concomitant with the release of the mf from the uterus, as it is not present at significant levels in developing mf (Figure 5.27), but is detectable in mf 6 hours after release (Figure 5.28). The culture of adult worms and the collection of mf allowed the manipulation of factors that may influence the expression of *mmc-1*. Variation of incubation temperature did not affect the expression of *mmc-1* in mf (Figure 5.29). It was predicted that *mmc-1* expression may have been affected by temperature as one of the major differences in the culture conditions used in this study was the manipulation of temperature, also *mmc-1* was previously isolated from a heat shock cDNA library produced from mf incubated at 41°C for two hours (section 3.4.5) [80]. The apparent high level of *mmc-1* mRNA in the heat shock library may be due to steady state expression of this transcripts at elevated temperatures when many other genes are down regulated following temperature stress [216]. The presence of serum in culture medium has been shown to influence the expression of excreted proteins of *B. malayi* [217]. However in this study the culture of adult parasites in the serum-free medium did not effect the level of mRNA expression seen in the released mf (Figure 5.30). Also the expression of *mmc-1* was not affected by absence of glucose from the medium or the absence of both serum and glucose (Figure 5.31).

It was hoped that the isolation of the up-stream region of *mmc-1* may have given an insight into possible factors involved in the regulation of *mmc-1*, unfortunately the preliminary attempts conducted here were unsuccessful. It was

assumed that in the initial screen the *mmc-1* probe had hybridised to a region of similarity which did not hybridise at the higher stringency of the tertiary screen. In retrospect the conditions used to screen the library may have been less than optimal. When genomic DNA was analysed by Southern blot (section 5.2.6) a degree of cross-reactivity was seen at low stringency (50°C, 2 X SSC, 0.1 % SDS washes, see Figure 5.16) which was not seen after washing at higher stringency (50°C, 1 X SSC, 0.1 % SDS, see Figure 5.17). In light of this the screen may have been less stringent than was required to isolate the *mmc-1* fragment by homologous screening. The lack of positively hybridising plaques may also relate to the length of probe used. As the probe is very short compared to the length of the lambda clone, the region of hybridisation would be limiting and would therefore require an increased hybridisation time.

Although the modification of the culture medium did not produce a visible difference in the expression of *mmc-1* mRNA it did effect mf behaviour. Mf in standard culture conditions (RPMI + 10 % FCS + 1 % glucose) exhibit very vigorous motility that resembles mf isolated *ex vivo* from an infected jird. In the minus glucose medium the mf were very sluggish and showed little motility as is seen in mf in the flight muscles of the mosquito host [9]. The motility of *B. pahangi* mf has been shown to be dependent on the presence of glucose as the culture of mf in glucose-free medium leads to a cessation of motility that can be reversed by the addition of glucose [218]. The availability of glucose may reflect the lack of motility of the parasite when in the mosquito flight muscles [9]. It has been shown that *B. patei* mf developing in the flight muscles of *Aedes togoi* do not incorporate significant amounts of glucose, and that parasitised muscle cells incorporate less glucose than

non-parasitised cells [219]. *In vitro* the incorporation of glucose is believed to be by a trans-cuticular route and uptake is only evident in viable worms and is therefore likely to be an active process [220]. How the glucose in the medium is exhausted is not clear, the uptake of glucose by *B. pahangi* is approximately equal in adult females (6.11 µg/mg wet body weight/hour) compared to adult males (7.75 µg/mg wet body weight/hour) [220] however the mf of *B. pahangi* can metabolise glucose up to 14 times faster than adults [221].

The number of mf released during the culture period was observed to drop in the glucose-free medium (Figure 5.32). This is likely to be due to the exhaustion of the energy source from the medium. In culture it has been shown that 13-14 % of available glucose is metabolised to trehalose and is used as a source of stored energy [222]. If adult *B. pahangi* are cultured and fed glucose at less than 0.4 mg/hour no trehalose is detectable as it is metabolised to lactate and succinate [223]. At the outset of the experiment the RPMI + 10 % FCS + 1 % glucose medium contained 68 mM glucose (see Table 5.5), therefore 10 ml of medium would contain 122.4 mg of glucose. Using the rates of glucose metabolism of Howells and Chen (1981) [220] (adult females 6.11 µg/mg wet body weight/hour, adult males 7.75 µg/mg wet body weight/hour) a culture of 50 males and 50 females would metabolise a total of 0.1768 mg glucose/hour (average wet weight of female = 0.495 mg, average wet weight of male = 0.066 mg [220]). Therefore even after 72 hours the amount of glucose would not be limiting ($0.1768 \times 72 = 12.73$ mg glucose metabolised), although this does not take into account the amount of glucose that would be incorporated by the released mf. The glucose free medium contained no detectable glucose (Table 5.5) therefore the only available energy source would be stored carbohydrates such as

trehalose which may become exhausted in the first 24 hours leading to the slowing of mf release seen in the later periods of culture (Figure 5.32).

In *C. elegans* egg laying is cyclical and related to environmental conditions [224]. *C. elegans* hermaphrodites have periods of inactivity of the uterus leading to reduced egg laying, whereas in periods of activity the uterus is stimulated to contract causing the expulsion of eggs. The contractions of the uterus are stimulated by neurons that respond to the presence of a food source. In wild type *C. elegans* the lack of a bacterial lawn reduces the release of eggs by reducing contraction of the muscles of the vulva, this behaviour is not seen in mutants lacking the *flp-1* gene which encodes a neuropeptide [224]. This type of behaviour is likely to be an example of adaptation to maximise survival, such that large numbers of eggs are only released when the environment is capable of supporting them. It is possible that the lack of mf release seen in the glucose-free medium may be an evolutionary strategy to reduce the release of mf in an unsuitable environment. The glucose concentration in the lymph of the thoracic duct of man has been reported to be between 95 and 140 mg/ml and follows that of the blood, which is tightly regulated by the pancreas [225]. Therefore adult parasites in the lymphatics of an animal host are in a relatively unchanging environment rich in glucose which may lead to the continuous release of the mf. The reduction in mf observed in glucose-free medium *in vitro* may be a limitation of the culture systems. Other factors may be involved in this process, e.g. mating behaviour and host factors. The release of the mf of *O. volvulus* shows a cyclical pattern of release, although this is thought to be related to fertilisation of females by "wandering males" [226]. The mating of *Brugia spp* has not been documented and so it is difficult to speculate if this type of behaviour occurs. The

possibility that host factors may influence the release of progeny is based on studies of *S. mansoni* where the cytokine TNF- α produced by the host, was found to be required for egg production [131]. Infection of SCID mice was found to result in > 70 % reduction in the amount of egg production compared to infected BALB/c mice. *In vitro* the numbers of eggs produced was increased by recombinant human TNF- α in a dose dependent manner [131]. Could a requirement for host signals to stimulate production of mf be present in *B. pahangi*? Seasonal periodicity has been reported in *B. malayi*, where the numbers of mf in the peripheral blood appear to mirror the seasonal prevalence of the mosquito vector [37]. If this is due to a seasonal variation in the production mf, a possible mechanism for the control of mf release could be that host reactions to the bite of a mosquito or to infecting L3 parasites, results in immune stimulation and cytokine production that stimulates the adult worms to produce increased numbers of mf. Therefore during the wet season when mosquitoes are abundant and biting rates are increased the release of mf is also increased to maximise the chance of infection.

On the basis of the analysis conducted it appears that *mmc-1* is expressed to coincide with the release of the mf from the adult female. Therefore some factor other than temperature, serum or glucose to which the mf are exposed to in the bloodstream may be responsible for triggering expression. Alternatively the release of the mf from the uterus may provide mechanical stimulation for initiation of *mmc-1* expression.

As *mmc-1* appeared to be specific to the mf stage of the *Brugia* life-cycle it may function either in the mammalian host or in the infection event in the mosquito. The evidence that *mmc-1* is expressed only in the mf stage of *Brugia* species raises a

number of questions, is *mmc-1* involved in the immuno-modulation in the mammalian host?; is *mmc-1* secreted from the worm as the nucleotide sequence would predict?; where is the protein located and does this give an insight to its possible function? and is the *mmc-1* protein seen in any other life cycle stage or is it also specific to the mf stage? The following Chapter aims to address some of these questions.

CHAPTER SIX

6.1 Introduction

In order to investigate the functions of the *mmc-1* gene product a recombinant MMC-1 protein was produced by expression in *E. coli*. The recombinant protein was produced using the pMal protein fusion and purification system (New England Biolabs). The purified protein was used in immunoassays to determine if MMC-1 is a natural antigen in both human and animals infected with *Brugia* species. The protein was also used to raise MMC-1 anti-serum in both rabbits and mice. The anti-sera were then used to identify the native protein by western blotting and investigate the localization of the MMC-1 protein in whole worms by IFAT localisation.

6.2 Production and purification of recombinant MMC-1 protein.

6.2.1 Cloning of *mmc-1* into MBP-p2 expression vector.

The ORF of *mmc-1* was amplified by PCR and cloned into the MBP expression vector. PCR of mammalian mf cDNA using two gene specific primers *mmc-1*ExF1 and *mmc-1*ExR1 resulted in a band of predicted size (266 bp) marked I Figure 6.1. The 266bp band was digested with *Bam* HI and *Pst* I, gel purified and ligated into the MBP vector. Three transformation positive clones, as determined by α -complementation, were sequenced using the *MalE* primer (New England Biolabs). All three clones possessed the predicted sequence and were in the correct orientation and correct frame downstream of the *MalE* gene to allow accurate expression .

6.2.2 Expression and purification of MBP-MMC-1 fusion protein.

A pilot experiment was conducted to determine if the clone could be induced to express the recombinant protein. An isolated colony was inoculated into LB-amp,

Figure 6.1 PCR of *mmc-1* ORF with *mmc-1ExF1* and *mmc-1ExR1* primers.

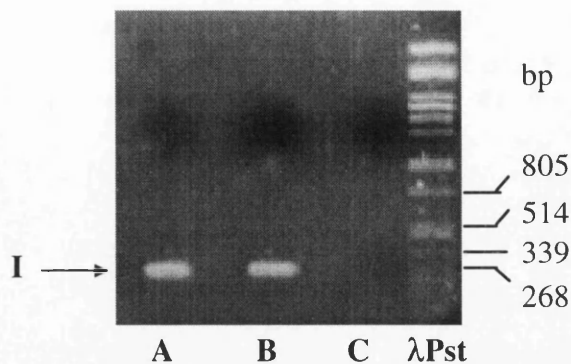


Figure 6.1

The open reading frame of the *mmc-1* gene was amplified by PCR using *mmc-1ExF1* and *mmc-1ExR1* primers. An aliquot of mammalian-derived mf cDNA was amplified under the following conditions: thirty cycles of 94°C 1 minute, 50°C 1 minute, 72°C 1 minute. A specific band of predicted size (266 bp) marked I was produced. The band was purified and cloned into pCR2.1 TA vector (Invitrogen).

- A = 1μl of mammalian mf cDNA, *mmc-1ExF1* and *mmc-1ExR1* primers.
B = 0.5 μl of mammalian mf cDNA, *mmc-1ExF1* and *mmc-1ExR1* primers.
C = No template DNA control, *mmc-1ExF1* and *mmc-1ExR1* primers.
λPst = *Pst* I digested λ DNA molecular size markers.

grown overnight at 37°C and induced under standard conditions. Samples of induced and uninduced samples were separated on a 12.5 % acrylamide SDS gel. Figure 6.2 shows the induction of the recombinant protein. The induced band had a predicted size of 51.8 kDa (42.5 kDa MBP + 9.3 kDa MMC-1), and a band of appropriate size marked I, can be seen in the induced lanes. The level of induction was relatively low and so expression was attempted in Topp cells (Stratagene) to optimise expression. Uninduced and induced samples were produced as described above for DH5α cells and separated as before (Figure 6.3). A slight increase in the level of induction of a band of the appropriate size can be seen in the induced lanes. A pilot experiment was carried out to purify the recombinant protein. The results of this experiment are shown in Figure 6.4. The MBP-MMC-1 protein is marked I. The gel shows that a small fraction of the fusion protein is contained in the insoluble fraction (lane C) whilst the majority is in the soluble fraction and can be purified from contaminating proteins by affinity purification on an amylose column (lane D). A large scale production of the fusion protein was conducted following the manufacturers protocol.

The crude extract obtained from the large scale expression was loaded onto an amylose column and washed to remove contaminating proteins. The MBP-MMC-1 fusion protein was eluted in 3 ml aliquots by washing with column buffer containing maltose, as detailed in methods. The results of the elution are shown in Figure 6.5. The MBP-MMC-1 protein is marked I. The MMC-1 protein was cut from MBP using Factor Xa (New England Biolabs) (2.9.6). Figure 6.6a shows an acrylamide gel with the cut MMC-1 protein of predicted size, 9.3 kDa, labelled I, the MBP moiety marked II, and a small proportion of the fusion protein remaining uncut, III. To purify the MMC-1 protein from the remaining contaminating bacterial

Figure 6.2 Induction of MBP-MMC-1 fusion protein expression in DH5 α competent *E. coli* cells.

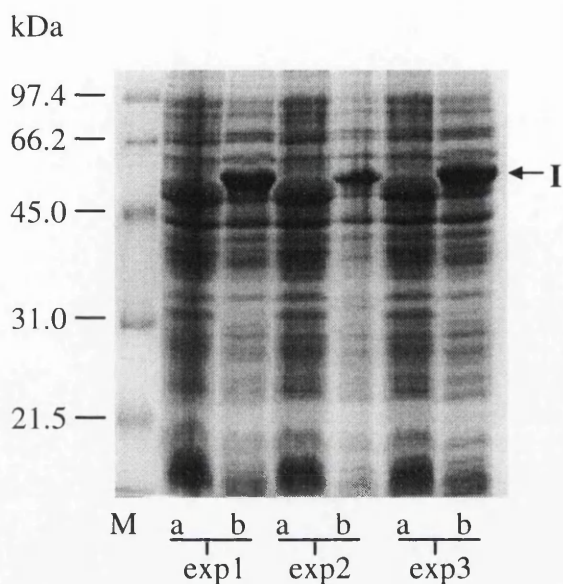


Figure 6.2

Three separate MBP-MMC-1 plasmids (exp 1-3) were transformed into DH5 α competent *E. coli* cells and the fusion protein was induced to express by addition of IPTG. An SDS sample cocktail extract of uninduced and induced cells was analysed by SDS-PAGE on 12.5 % gels. An induced band of predicted size (51.8 kDa) corresponding to the MBP-MMC-1 fusion protein can be seen (marked I) in each of the three induced lanes.

a = Uninduced protein sample.
b = Induced protein sample.
M = Low molecular weight protein standards.

Figure 6.3 Induction of MBP-MMC-1 fusion protein expression in Topp competent *E. coli* cells.

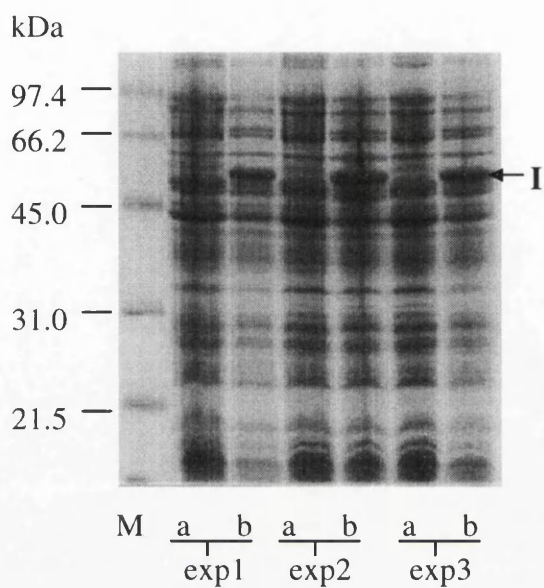


Figure 6.3
The three plasmids, exp 1-3, were transformed into Topp cells (Stratagene) and the fusion protein was induced to express by addition of IPTG. An SDS sample cocktail extract of uninduced and induced cells was analysed by SDS-PAGE on 12.5 % gels. An induced band of predicted size (51.8 kDa) corresponding to the MBP-MMC-1 fusion protein can be seen (marked I) in each of the three induced lanes.

a = Uninduced protein sample.
b = Induced protein sample.
M = Low molecular weight protein standards.

Figure 6.4 Pilot experiment of MBP-MMC-1 expression and purification.

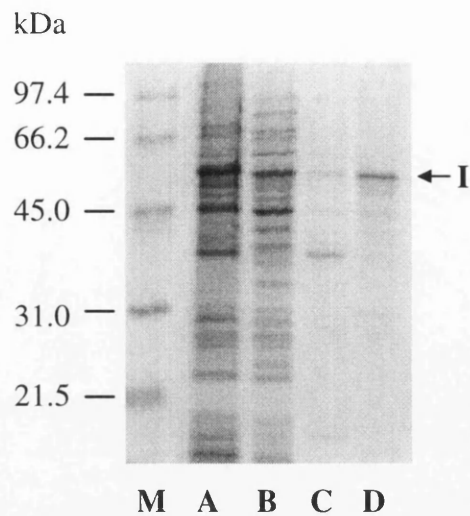


Figure 6.4

The MBP-MMC-1 fusion was induced to express in Topp cells and purified following the manufacturers protocol. The purification of the fusion protein, marked I, can be followed through the procedure. The proteins were analysed by SDS-PAGE on a 12.5 % gel .

- A = Total induced protein (sonicated induced cells).
- B = SDS sample cocktail soluble.
- C = Insoluble fraction.
- D = Amylose purified protein.
- M = Low molecular weight protein standards.

Figure 6.5 Affinity purification of MBP-MMC-1 fusion protein.

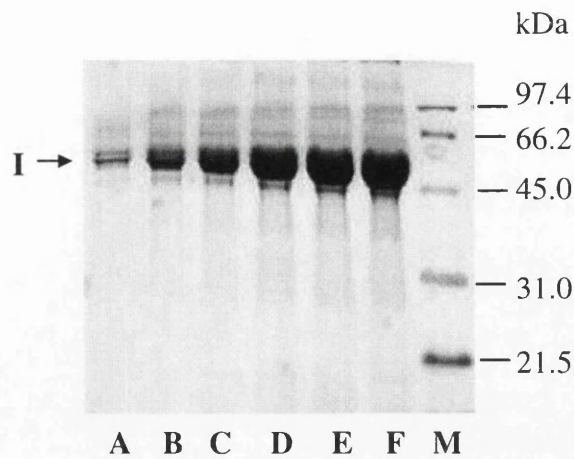


Figure 6.5
Induced expression in DH5 α cells, sonicated in column buffer (20 mM Tris-Cl, 200 mM NaCl 1 mM EDTA) to lyse cells. Cells collected by centrifugation at 10000g 15 minutes. The soluble protein supernatant was resuspended in column buffer and passed over an amylose column. The fusion protein was eluted from the column with column buffer containing 20 mM maltose and collected in 3 ml aliquots. 10 μ l aliquots were analysed on a 12.5 % acrylamide gel. A-F are sequential aliquots from the amylose cloumn showing an increase of protein eluted (marked I) with subsequent fractions.

M = Low molecular weight protein standards.

Figure 6.6a

The affinity purified MBP-MMC-1 fusion protein was digested for 6 hours at 23°C with Factor Xa (New England Biolabs) to liberate the MMC-1 recombinant protein. The digested proteins were size separated on a 12.5% acrylamide gel. The MMC-1 protein is marked I and the released MBP moiety is marked II. A small proportion of the fusion protein remained uncut (marked III).

A = Affinity purified uncut MBP-MMC-1 fusion protein.

B = Purified MBP-MMC-1 cut by Factor Xa.

C = Purified MBP-MMC-1 cut by Factor Xa.

M = Low molecular weight protein standards.

Figure 6.6b

The liberated mmc-1 protein was excised from the gel and electro-eluted for further purification.

A = 10 µl of eluted MMC-1 recombinant protein.

B = 30 µl of eluted MMC-1 recombinant protein.

uM = Ultra low molecular weight protein standards.

M = Low molecular weight protein standards.

Figure 6.6a Liberation of recombinant mmc-1 by digestion with Factor Xa.

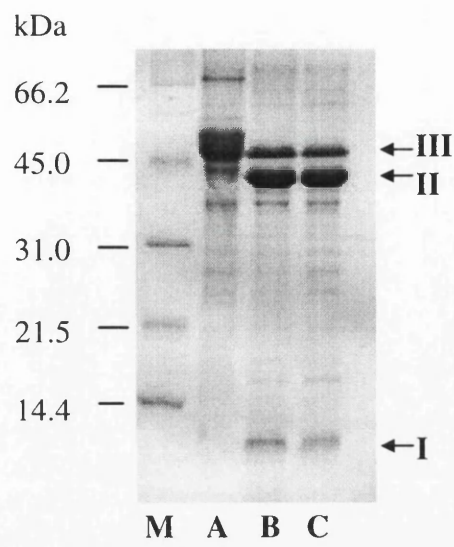
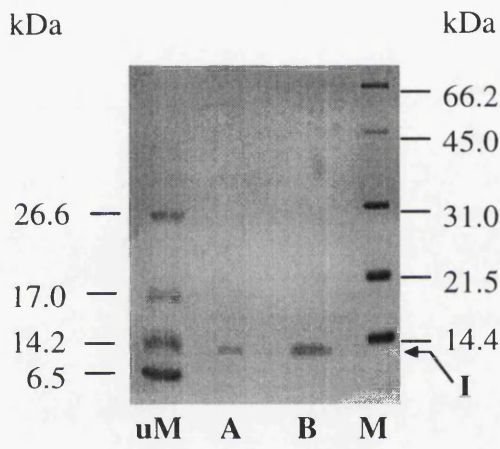


Figure 6.6b



proteins, the MMC-1 band was excised from the gel washed in H₂O and stored at -20°C. For some experiments where purified MMC-1 was required, it was isolated from the acrylamide using an electro-eluter (Bio-Rad, section 2.9.6). The eluted band was re-analysed by SDS-PAGE (Figure 6.6b).

6.2.3 Raising MMC-1 anti-serum.

The recombinant MMC-1 protein in acrylamide gel was frozen in liquid nitrogen and pulverized. This was then used to produce polyclonal antiserum by immunisation of mice and rabbits, as described in section 2.10.

Two rabbits were immunised and boosted twice following which the antiserum was tested along with the appropriate pre-bleeds. Figure 6.7 shows a western blot highlighting the positive recognition of the recombinant MMC-1 protein by the immune serum from one of the rabbits (718) but not by the other (720). MMC-1 was not recognised by the pre-bleeds (lanes A and C). Also shown in Figure 6.7 (lane F) is the reactivity of a pool of immunised mouse serum (BALB/c). No recognition of MMC-1 was observed with control mouse serum (Lane E).

6.2.4 Detection of MMC-1 in different life-cycle stages.

To determine if the MMC-1 protein is present in life-cycle stages other than the mf, protein extracts were separated by SDS-PAGE transferred to nitrocellulose and the presence of MMC-1 detected by western blotting with the MMC-1 rabbit antiserum. SDS sample cocktail extracts of adult, L3 and mf were produced under standard conditions (2.11.2) and separated by SDS-PAGE. Figure 6.8 shows a Coomassie Blue stained gel to illustrate approximately equal loading of the protein

Figure 6.7 Testing the reactivity of MMC-1 antisera by Western blot.

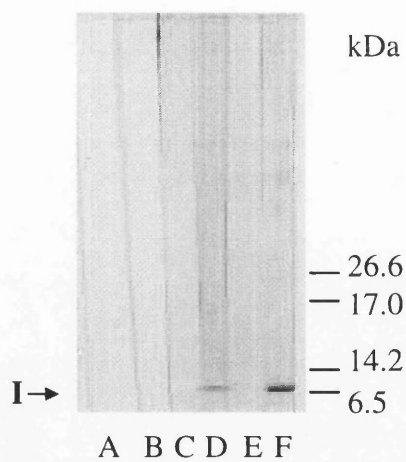


Figure 6.7

The recombinant MMC-1 protein separated on a 12.5 % acrylamide gel was immunoblotted and probed with either anti-MMC-1 or control serum from immunised rabbits or mice. The serum was used at a dilution of 1:500, goat anti-rabbit or anti-mouse alkaline phosphatase conjugate was at 1:6000 and the blot was developed with BCIP/NBT substrate.

- A = Rabbit R720 pre-immune sera.
- B= Rabbit R720 anti-MMC-1 sera.
- C = Rabbit R718 pre-immune sera.
- D = Rabbit R718 anti-MMC-1 sera.
- E = Pooled control BALB/c mouse sera.
- F = Pooled BALB/c anti-MMC-1 sera.

Figure 6.8 SDS-PAGE of L3, mf and adult extracts.

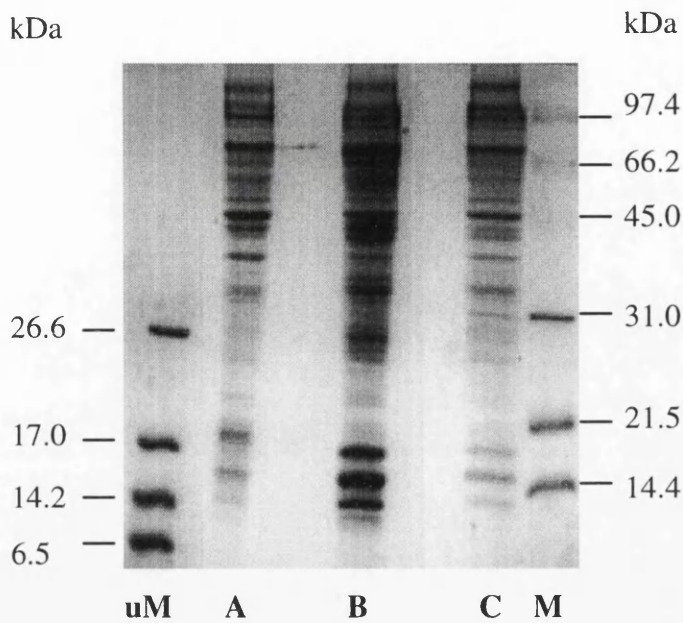


Figure 6.8

Approximately equal quantities of SDS-sample cocktail extracts of L3, mf or adult *B. pahangi* were separated on a 15 % acrylamide gel.

uM = Ultra low molecular weight protein standards.

A = SDS-sample cocktail extract from infective L3.

B = SDS-sample cocktail extract from mature mf.

C = SDS-sample cocktail extract from mixed sex adults.

M = Low molecular weight protein standards.

samples. A similar gel was then blotted and probed with the R718 MMC-1 antiserum or pre-immune R718 control serum. Two specific bands (approximately 12 kDa and 8 kDa) could be seen in the mf protein extract (Figure 6.9 lane D). The immune sera did not recognise any proteins in the L3 or adult lanes (Figure 6.9 lanes B and F, respectively).

6.2.5 Detection of MMC-1 in *B. pahangi* mf excreted/secreted products.

The MMC-1 predicted protein sequence contains a secretory leader and may therefore be secreted from the parasite. Experiments were undertaken to determine if the MMC-1 protein could be detected in the excreted/secreted (E/S) products collected from parasites cultured *in vitro*. Approximately 2×10^6 mf isolated from the peritoneal cavity of an infected jird were cultured aseptically for 24 hours in 20 ml of RPMI+ medium (RPMI 1640 + 10 % FCS, 1 % Glucose, 2 mM L-glutamine, 2.5 mM Hepes, 100u/ml penicillin, 100µg/ml streptomycin). Mf were removed by centrifugation and the medium concentrated 40 fold to approximately 500 µl, using a 5 kDa molecular weight cut-off concentration column (Vivaspin). The E/S products were re-suspended in SDS sample cocktail and separated by SDS-PAGE and visualised by Coomassie Blue staining (Figure 6.10). A number of bands were obvious, although as the culture medium contained FCS, serum proteins are likely to account for the predominant bands. The proteins were transferred to nitrocellulose by standard methods and tested for the presence of MMC-1 by western blotting. The antiserum did not recognise any proteins from the E/S products, even at high concentrations (1/50 dilution of rabbit 718 antiserum). In order to determine if any proteins could be detected when mf were cultured in serum-free medium, the culture

Figure 6.9 The recognition of MMC-1 in different life-cycle stages by Western blot.

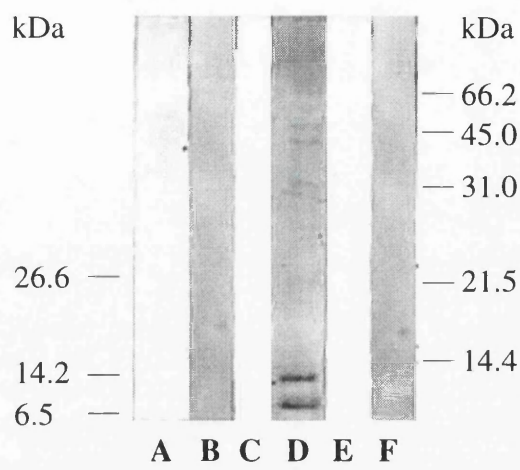


Figure 6.9

SDS-sample cocktail extracts of L3, mf and mixed sex adult *B. pahangi* were separated by SDS-PAGE on a 15 % acrylamide gel and immunoblotted. The resulting blot was probed with either anti-MMC-1 or pre-immune rabbit serum. The serum was used at a dilution of 1:500, goat anti-rabbit alkaline phosphatase conjugate was at 1:6000 and the blot was developed with BCIP/NBT substrate.

A = L3 SDS-sample cocktail extract, Rabbit R718 pre-immune sera.

B = L3 SDS-sample cocktail extract, Rabbit R718 anti-MMC-1 sera.

C = Mf SDS-sample cocktail extract Rabbit R718 pre-immune sera.

D = Mf SDS-sample cocktail extract Rabbit R718 anti-MMC-1 sera.

E = Mixed sex adult SDS-sample cocktail extract, R718 pre-immune sera.

F = Mixed sex adult SDS-sample cocktail extract, R718 anti-MMC-1 sera.

Figure 6.10 Coomassie Blue stained *B. pahangi* mf E/S products.

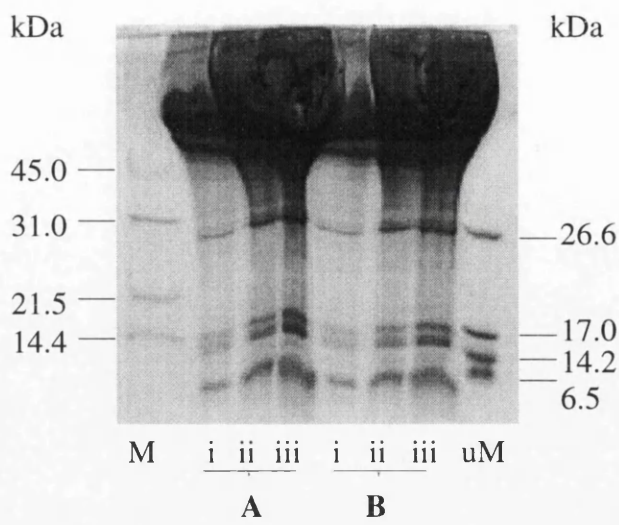


Figure 6.10

Approximately 2×10^6 mf were cultured for 24 hours in RPMI+ medium. The mf were removed by centrifugation and the collected E/S products were concentrated by centrifugation through a 5 kDa protein concentration column. Three aliquots from duplicate experiments were separated by SDS-PAGE on a 12.5 % acrylamide gel and stained with Coomassie Blue.

- M = Low molecular weight protein standards.
- uM = Ultra low molecular weight protein standards.
- i, ii and iii = 5, 10 and 20 µl of collected ES products.
- A and B are duplicate experiments.

and collection of E/S was repeated without FCS in the medium. However, no proteins could be detected in the E/S products of mf cultured in serum-free medium despite the 40-fold concentration and silver staining. Figure 6.11 shows a silver stained acrylamide gel of the concentrated E/S products. The lack of stained bands may be due to low a concentration of E/S products or may reflect the fact that in the absence of serum, the culture conditions are sub-optimal and result in a reduction in metabolism and hence secreted proteins. To determine if MMC-1 protein could be detected at low levels the E/S products were tested by western blotting. Again no reaction was observed between the MMC-1 anti-serum and the E/S products.

6.2.6 Metabolic labelling of mf and MMC-1 immunoprecipitation.

To investigate the synthesis of MMC-1 by the mf of *B. pahangi*, immunoprecipitation of ^{35}S methionine metabolically labelled mf proteins was attempted. Mf were labelled as described in Devaney *et al*, (1992) [41], except that the labelled proteins were extracted by homogenization in isoelectric focusing buffer (IEF) using a micropestle (Eppendorf). 1×10^6 precipitable counts were separated on a SDS-polyacrylamide gel which was subjected to fluorography and exposed to X-ray film for 8 weeks. The experiment was conducted on two separate occasions and both times no detectable protein could be precipitated (data not shown). However the extraction of total protein showed that the labelling of the proteins was successful. The inability to detect MMC-1 by immunoprecipitation may relate to the method used to extract the labelled proteins. Although the extract was diluted 20 fold in TBS prior to the addition of the antiserum, the presence of urea in the IEF lysis buffer (9.5 M) may have disrupted the antigen/antibody complexes.

Figure 6.11 Silver stained gel of *B. pahangi* mf E/S products.

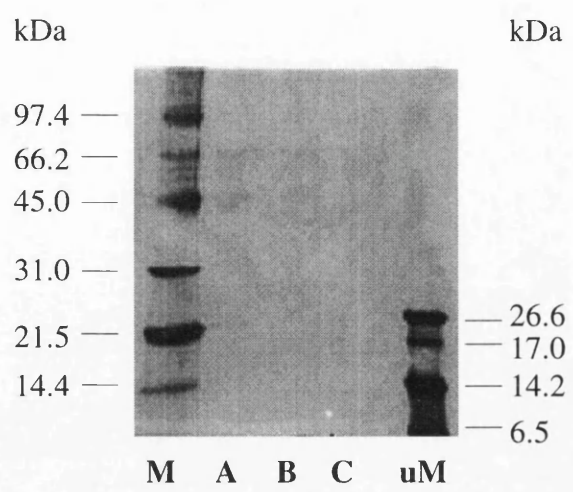


Figure 6.11
E/S products collected from *B. pahangi* mf cultured in serum free medium were concentrated 40-fold and separated by SDS-PAGE on a 10-20 % gradient acrylamide gel. The gel was stained with silver nitrate.

- M = Low molecular weight protein standards.
- uM = Ultra low molecular weight protein standards
- A = 10 µl of concentrated E/S products.
- B = 20 µl of concentrated E/S products.
- C = 30 µl of concentrated E/S products.

6.2.7 Localisation of the MMC-1 protein in *B. pahangi* mf.

The MMC-1 antiserum was used to localise the MMC-1 protein in the mf of *B. pahangi* by immunofluorescent analysis (section 2.14.1). Initially intact sheathed and exsheathed mf were tested to determine if MMC-1 was expressed on the surface of the sheath or the cuticle. The MMC-1 antibody was negative at all dilutions of antiserum tested (1/50-1/200) on both sheathed and pronase-exsheathed mf. The control pre-bleed was also negative with both samples (Figure 6.12a and 6.12b). In these experiments the only fluorescence observed was to the cut ends of worms that had been damaged during the procedure (Figure 6.12c). As MMC-1 was not present on the surface of intact or exsheathed mf, but appeared to be localised internally, experiments were carried out to investigate the internal localisation. For these experiments a technique used for *in situ* antibody labelling of whole *C. elegans* was adapted for use with *B. pahangi* mf. In this method the mf were first exsheathed and then partially digested by incubation overnight in BME solution (2.14.2) then 4 hours in collagenase buffer (115 digestion units/ml 2.14.2). Permeabilised whole worms were incubated with MMC-1 or control antibody at 1/100 dilution overnight at 4°C, washed and incubated in goat anti-rabbit FITC conjugate for 4 hours. In these experiments the fluorescence observed with the MMC-1 antiserum was distributed throughout the worm (Figure 6.13a). No reaction was seen with the control pre-immune rabbit sera (Figure 6.13b). The analysis did not localise MMC-1 to a particular tissue in the mf as the fluorescence was relatively equally distributed throughout the entire length of the worm and suggests that MMC-1 may be expressed in all cells of the worm.

Figure 6.12 Immunofluorescence analysis of MMC-1 localisation in sheathed and exsheathed mf of *B. pahangi*.

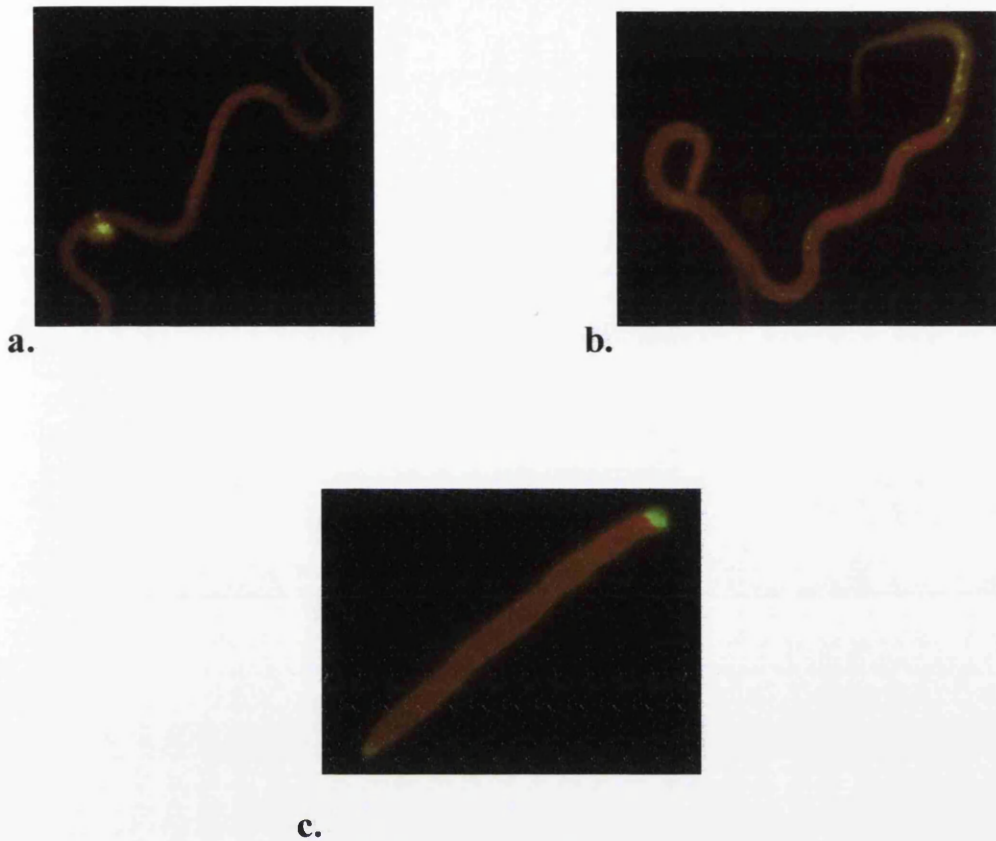


Figure 6.12
Approximately 1×10^5 mf were fixed in 4% PFA in PBS for 12 hours at 4°C then washed in PBS and incubated with anti-MMC-1 rabbit antiserum at 1/100 dilution, followed by goat anti-rabbit IgG FITC conjugate at 1/200 dilution (panels a and c). In some experiments, the sheath was removed by exposure to pronase enzyme (1mg/ml 10 minutes at room temperature) then reacted with the MMC-1 antisera as described above (panel b). Mf were viewed on a Olympus BX60 UV fluorescence microscope and photographed with a SPOT photo capture system. Magnification x 400.

- a = Sheathed mf, anti-MMC-1 1/100
- b = Exsheathed mf, anti MMC-1 1/100
- c = Fragment of sheathed mf, anti-MMC-1 1/100

Figure 6.13 Immunoflourescent localisation of MMC-1 anti-sera in partially digested mf.

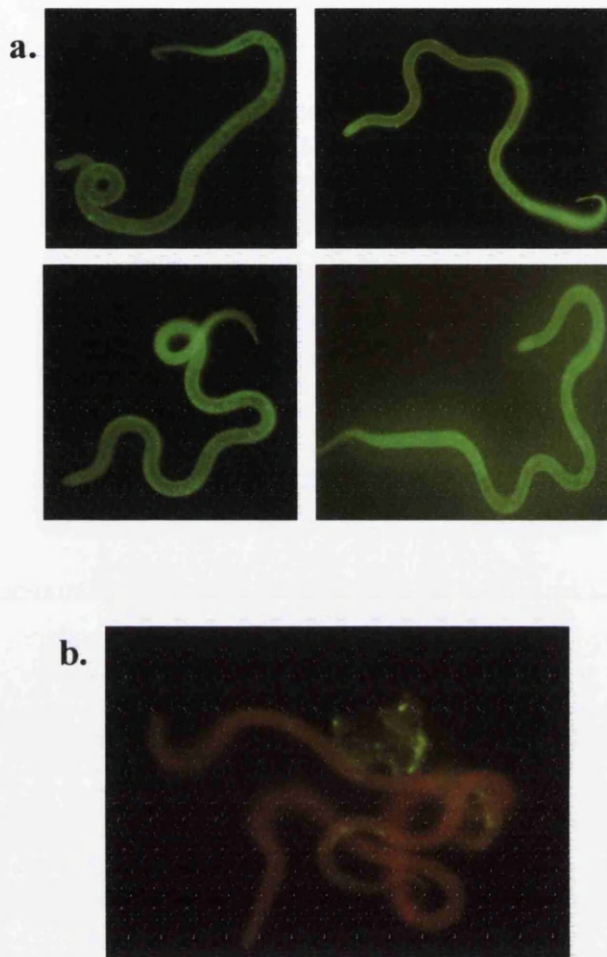


Figure 6.13

Approximately 1×10^5 pronase exsheathed mf were fixed in 4% PFA in PBS for 12 hours at 4°C. The mf were washed in PBS then subjected to a digestion protocol of overnight exposure to 5% BME at 37°C then a four hour incubation at 37°C in collagenase (115 digestion units/ml). Permeabilised mf were then incubated with anti-MMC-1 rabbit antiserum at 1/100 dilution, followed by goat anti-rabbit IgG FITC conjugate at 1/200 dilution. Mf were viewed on a Olympus BX60 UV fluorescence microscope and photographed with a SPOT photo capture system. Magnification x 400.

a = anti-MMC-1 rabbit serum (1/200 dilution).

b = control rabbit pre-immune serum (1/200 dilution).

6.2.8 Does MMC-1 have a role in the infection of mf in the mosquito vector?

As MMC-1 is expressed exclusively in the mf, one possibility is that the protein may be involved in the infection event of the mosquito. In order to investigate this hypothesis an IgG cut was prepared from the MMC-1 rabbit anti-serum (2.13.1). Mosquitoes were fed on blood containing 1.75×10^4 mf/ml supplemented with IgG of either MMC-1 anti-serum or an irrelevant rabbit antiserum (raised to cytidine deaminase, CDD) at 100 µg/ml (2.13.2). The development of the parasites at day 9 post-infection was assessed by dissection of individual mosquitoes into head, thorax and abdomen and counting the number of L3 parasites present in each part. The numbers of L3 were compared between mosquitoes fed on blood containing MMC-1 IgG, CDD IgG or no antibody. At day 9 p.i. in normal mosquito infections the parasites are infective L3 and are present in the head and mouthparts in readiness for the mosquito to mammal infection event, or have migrated throughout the haemocoel.

The results of two duplicate experiments are shown in Table 6.1. In experiment one there was no significant difference in the total numbers of L3s between the three groups. However when the data were analysed with respect to the location of the L3, there was a difference between the mosquitoes fed MMC-1 IgG and the other two groups. In the MMC-1 group a significantly increased proportion of the L3s were in the abdomen (51.5 %) compared to the other two groups (22%, control and 18 % cdd-IgG) $p < 0.001$ (Figure 6.14). Correspondingly in the two control groups, there were greater numbers of L3 in the head than in the abdomen. In the second experiment a significant difference was detected in the numbers of L3s in the three groups of mosquitoes. Those fed on MMC-1 IgG had the highest number of

Table 6.1

Aedes aegypti, strain (Ref^m) mosquitoes were fed infected rabbit blood (1.75×10^4 mf/ml) supplemented with either 100µg/ml CDD IgG or 100µg/ml MMC-1 IgG for one hour, the blood replaced and again fed for one hour. Fed females were isolated in a netted cage and maintained for 9 days. At day 9 p.i the mosquitoes were dissected into head, thorax and abdomen and the numbers of infective larvae were recorded. The data in the table shows the recovery of L3 parasites from ten dissected mosquitoes from each group.

Table 6.1 Effect of the inclusion of mmc-1 IgG in infected blood on the development of L3 stage parasites in the mosquito host.

Experiment #1

Control	Head	Thorax	Abdomen	Totals
1	8	1	6	15
2	23	7	6	36
3	14	0	1	15
4	11	3	3	17
5	4	12	23	39
6	6	1	0	7
7	19	1	5	25
8	12	1	0	13
9	7	0	2	9
10	11	0	8	19
Mean	11.5	2.6	5.4	195

Experiment #2

Control	Head	Thorax	Abdomen	Totals
1	8	1	1	10
2	1	2	1	4
3	0	2	1	3
4	2	4	11	17
5	1	1	2	4
6	1	4	2	7
7	4	5	1	10
8	1	2	2	5
9	3	7	2	12
10	3	2	1	6
Mean	2.4	3	2.4	78

<i>mmc-1</i>	Head	Thorax	Abdomen	Totals
1	15	1	4	20
2	0	6	28	34
3	12	6	2	20
4	9	2	4	15
5	1	2	35	38
6	0	4	11	15
7	4	12	29	45
8	0	3	19	22
9	7	1	0	8
10	1	12	19	32
Mean	4.9	4.9	15.1	249

<i>mmc-1</i>	Head	Thorax	Abdomen	Totals
1	1	2	9	12
2	0	6	5	11
3	3	5	5	13
4	3	3	7	13
5	8	8	10	26
6	2	4	7	13
7	3	3	3	9
8	8	7	8	23
9	2	6	6	14
10	4	4	5	13
Mean	3.4	4.8	6.5	147

<i>cdd-1</i>	Head	Thorax	Abdomen	Totals
1	14	4	6	24
2	19	1	3	23
3	19	2	6	27
4	20	0	1	21
5	7	5	2	14
6	17	8	4	29
7	9	1	2	12
8	22	4	2	28
9	9	1	6	16
10	16	6	6	28
Mean	15.2	3.2	3.8	222

<i>cdd-1</i>	Head	Thorax	Abdomen	Totals
1	6	2	4	12
2	2	2	3	7
3	2	3	4	9
4	2	2	7	11
5	1	2	0	3
6	4	5	9	18
7	7	4	1	12
8	1	2	3	6
9	3	2	3	8
10	2	3	4	9
Mean	3	2.7	3.8	95

Figure 6.14 Effect of the inclusion of MMC-1 IgG in infected blood on the development of L3 stage parasites in the mosquito host.

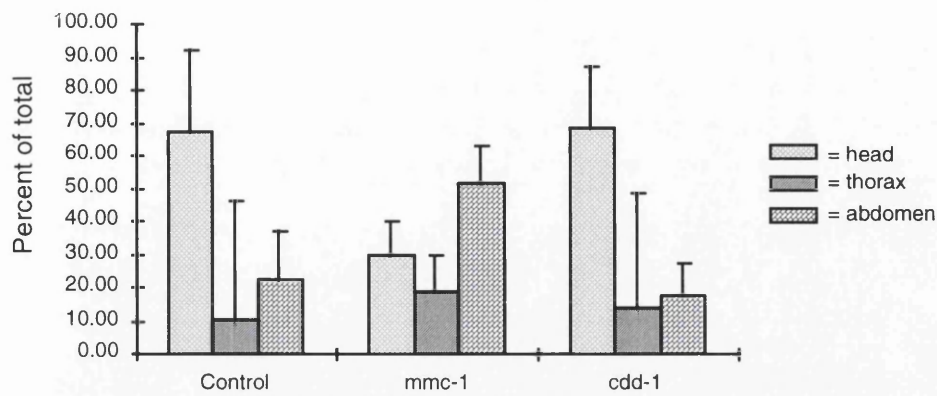


Figure 6.14
The data presented for experiment # 1 in Table 6.1 are shown above as percentage recoveries (mean \pm standard deviation) of L3 from head, thorax and abdomen for the three groups of mosquitoes.

L3 (mean 14.7 L3 per mosquito $p < 0.01$ compared to no IgG control, and $p < 0.1$ compared to CDD group). Mosquitoes fed on CDD IgG contained a mean of 9.5 L3 per mosquito while the no IgG control group contained a mean of 6.8 L3 per mosquito). As in experiment one there was an increased proportion of L3 in the abdomen of the mosquitoes fed MMC-1 antibody, but on this occasion the difference did not reach statistical significance. It is noteworthy that in experiment two, the overall numbers of parasites developing in the mosquito were much reduced compared to experiment one.

6.2.9 Testing the antigenicity of MMC-1.

Using ELISA a number of experiments were conducted to investigate the antigenicity of the recombinant MMC-1 protein in immunised or mf infected mice. BALB/c mice infected with different life-cycle stages of *B. pahangi* or immunised with purified MMC-1 were tested for the presence of MMC-1 specific IgG. The presence of anti-MMC-1 IgG subclasses in human sera from individuals infected with *B. malayi* was also investigated in collaboration with Dr Xingxing Zang, University of Edinburgh.

6.2.9.1 The reactivity of MMC-1 with immunised or mf infected mouse serum.

A group of five BALB/c mice, immunised four times with gel purified MMC-1 protein were bled out and the serum was isolated using standard protocols (2.10). The serum from five immunised mice was compared with serum from mice injected with Freund's incomplete adjuvant alone. Serum from each individual animal was tested by ELISA against the MBP-MMC-1 fusion protein or against MBP alone. The

results of a typical test are shown in Table 6.2 whilst Figure 6.15 shows a graphical representation of the data (OD value obtained with MMC-1 antisera minus the MBP background obtained with each sample). Immunisation with MMC-1 results in a specific IgG response compared to mice given Freund's incomplete adjuvant alone. The OD values obtained with individual sera were significantly greater to MMC-1 than to MBP ($p < 0.01$ by ANOVA).

The next set of experiments used the same assay to determine if animals infected with mf or L3 of *B. pahangi* would produce an IgG response specific to MMC-1. Serum from mice taken twelve days post-infection with either 1×10^5 mf, 50 L3 or an equivalent volume of HBSS as a control (all intravenous infection, provided by Richard O'Connor, University of Glasgow), was assessed for reactivity to MMC-1 as described above. Table 6.3 shows the OD readings for individual mice reacted with MBP-MMC-1 or MBP alone, while the data are shown graphically in Figure 6.16. The group infected with mf do show a minimal reaction to MMC-1 which is significantly different to the other groups.

6.2.9.2 Reactivity of MMC-1 with human sera from *B. malayi* infected individuals.

To determine if a humoral response to MMC-1 could be detected in human sera from individuals infected with *B. malayi* a similar set of experiments was conducted in collaboration with Dr Xingxing Zang (University of Edinburgh). In these experiments the profile of reactivity to MMC-1 of 16 mf positive (mf +) subjects, 16 mf negative subjects (mf -, determined by the lack of circulating mf by Nucleopore filtration of venous blood) and 6 European normal subjects was

Table 6.2 ELISA to detect MMC-1 IgG.

	MBP-MMC-1		MBP	
	Adjuvant control	MMC-1 immunised	Adjuvant control	MMC-1 immunised
	0.116	0.418	0.151	0.106
	0.124	0.563	0.143	0.124
	0.118	0.343	0.115	0.140
	0.125	0.666	0.081	0.137
	0.137	0.778	0.145	0.080
	Mean	0.124	0.554	0.127
	Standard deviations	0.008	0.177	0.029
				0.025

Table 6.2

Serum from either MMC-1 immunised mice or adjuvant only immunised mice were tested for the presence of MMC-1 specific IgG. The MBP-MMC-1 fusion protein was used to coat the ELISA plate (250 ng/well) and each serum was reacted in duplicate at a 1/100 dilution. Each serum was also reacted against MBP alone (250 ng/well). Bound antibody was detected using goat anti-mouse IgG horseradish peroxidase conjugate at 1/1500 dilution and developed using a TMB substrate. The plates were read after 2 minutes at 620nm. The results are presented as OD values for each individual animal, and a mean and standard deviation for each group.

Figure 6.15 Immunisation of mice with MMC-1 produces a specific IgG response.

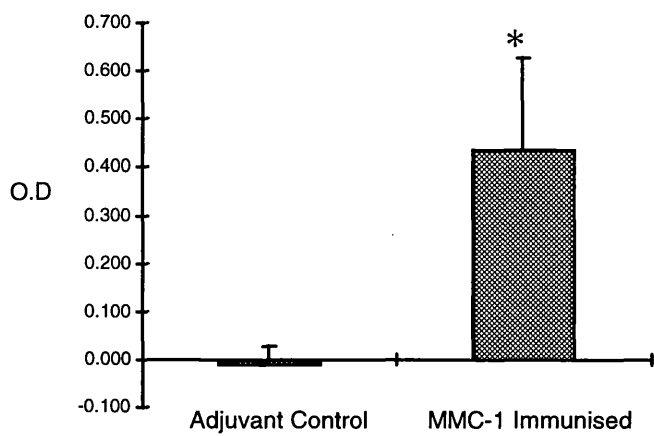


Figure 6.15
IgG responses of sera from groups of five mice immunised with recombinant MMC-1 or given Freund's incomplete adjuvant alone. The data shown in Table 6.2 were plotted using OD readings for MBP-MMC-1 reactivity minus the MBP background reactivity of individual mice. The graphs shows the mean of each group and standard deviations within each group.

Table 6.3 ELISA to detect MMC-1 IgG in mf or L3 infected mice.

MBP-MMC-1			
	HBSS control	mf infected	L3 infected
	0.091	0.147	0.075
	0.103	0.127	0.112
	0.091	0.168	0.098
	0.088	0.199	0.131
		0.152	0.103
Mean	0.093	0.159	0.104
Standard deviations	0.007	0.014	0.024

MBP			
	HBSS control	mf infected	L3 infected
	0.166	0.097	0.105
	0.098	0.090	0.146
	0.101	0.109	0.123
	0.115	0.125	0.164
		0.099	0.123
Mean	0.120	0.104	0.135
Standard deviations	0.032	0.014	0.026

Table 6.3
Mice were infected with 1×10^5 mf, 50 L3 or an equivalent volume of HBSS by the i.v route. At day 12 p.i. mice were bled out and serum used in an ELISA . The MBP-MMC-1 fusion protein was used to coat the ELISA plate (250 ng/well) and each serum was reacted in duplicate at a 1/100 dilution. Each serum was also reacted against MBP alone (250 ng/well). Bound antibody was detected using goat anti-mouse IgG horseradish peroxidase conjugate at 1/1500 dilution and developed using a TMB substrate. The plates were read after 2 minutes at 620nm. The results are presented as OD values for each individual animal, and a mean and standard deviation for each group.

Figure 6.16 Production of MMC-1 specific IgG in mf and L3 infections at 12 p.i.

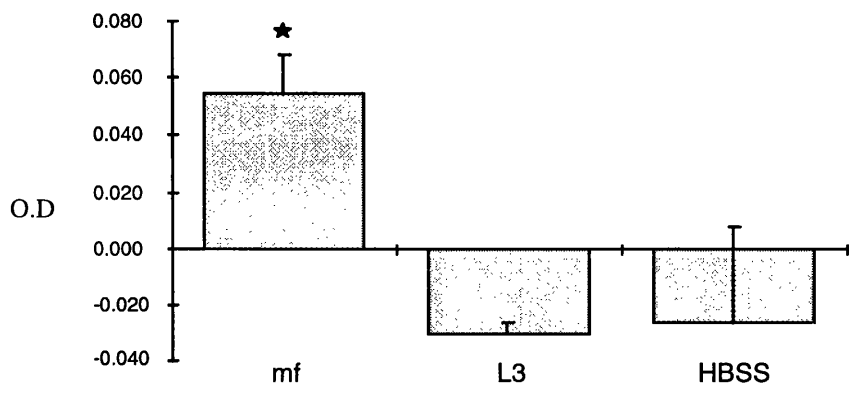


Figure 6.16
IgG responses of sera from groups of five mice infected with 1×10^5 mf, 50 L3 or HBSS. The data shown in Table 6.3 were plotted using OD readings for MBP-MMC-1 reactivity minus the MBP background reactivity of individual mice. The graphs shows the mean of each group and standard deviations within each group.

determined. The serum samples were obtained from the Renegat district of Sumatra, Indonesia an area endemic for *B. malayi*. The same samples have been used in previous studies [227, 228]. In the experiments reported here, subclass specific antibodies were used to investigate the recognition of MMC-1 by human sera (section 2.15.8). Table 6.4 shows the data obtained from this set of experiments presented as OD values against the fusion protein or to MBP alone. The reactivity to MMC-1 minus the MBP background is shown in Figure 6.17. The data show that both the mf positive and mf negative samples exhibit elevated levels of IgG1 and IgG3 to MMC-1 compared to the European control subjects. When ANOVA tests were conducted both the mf + and mf - groups showed significantly more IgG3 than the European normal group whilst none of the groups showed significant variance in IgG1 levels. No significant difference was seen in the amount of IgG1 or IgG3 between the mf + and mf - groups.

6.2.10 T cell responses to MMC-1.

Five BALB/c mice were immunised on two occasions with MMC-1 in Freund's incomplete adjuvant. For comparison, groups of five mice were immunised with either 100 µg mf antigen in Freund's incomplete adjuvant or with adjuvant alone. Spleens were removed and splenocytes re-stimulated *in vitro* with either electro-eluted MMC-1, mf antigen or adult antigen (section 2.15). The proliferation of T-cells following re-stimulation was measured by incorporation of ³H thymidine [96]. In addition secretion of cytokines IL-4, IL-5, IL-10 and IFN-γ by the cells was also tested using a capture ELISA as detailed in Osborne *et al* (1996) [96].

Table 6.4

Serum from subjects characterised as either, asymptomatic mf-, mf + or European normal controls were tested for the presence of MMC-1 specific IgG subclasses. The MBP-MMC-1 fusion protein was used to coat ELISA plates (200 ng/well diluted in 0.06 M carbonate buffer) each serum was reacted in duplicate at a 1/100 dilution (diluted in PBS/0.05 % Tween 20). Each serum was also reacted against MBP alone (200 ng/well). Bound antibody was detected using isotype-specific mouse monoclonal antibody (anti-IgG1 1/4000, anti-IgG2 1/2000, anti-IgG3 1/1000 and anti-IgG4 1/4000 diluted in PBS/0.05 % Tween 20 obtained from SkyBio). Bound IgG was recognised by peroxidase-conjugated rabbit anti-mouse Ig (1/1500) and developed using ABTS substrate (KPL Biotechnology). The plates were read after 2 minutes at 405nm. The results are presented as OD values for each individual, and a mean and standard deviation for each group.

Table 6.4 IgG subclass recognition of mmc-1 in human *B. malayi* infections.

mf positive MI/ml blood	IgG1	IgG1	IgG2	IgG2	IgG3	IgG3	IgG4	IgG4
	mmc-1	MBP	mmc-1	MBP	mmc-1	MBP	mmc-1	MBP
337	0.964	0.547	0.278	0.2866	1.366	0.829	0.240	0.145
194	0.820	0.706	0.147	0.2633	2.071	1.485	0.358	0.267
1303	0.568	0.677	0.131	0.2822	0.653	0.191	0.274	0.190
293	0.752	0.420	0.165	0.2700	1.273	0.371	0.211	0.124
241	0.529	0.263	0.279	0.2777	0.312	0.167	0.230	0.155
40	0.414	0.228	0.146	0.1200	1.961	0.192	0.172	0.151
45	0.623	0.363	0.199	0.1144	1.770	0.975	0.184	0.168
835	0.651	0.683	0.174	0.2666	0.944	0.512	0.212	0.176
175	1.470	0.711	0.323	0.3069	1.111	0.412	0.244	0.211
599	1.052	1.446	0.139	0.2677	1.530	1.213	0.258	0.278
681	0.352	0.380	0.276	0.2422	1.880	0.362	0.202	0.135
393	0.322	0.345	0.182	0.2759	0.860	0.968	0.276	0.192
730	0.617	0.280	0.176	0.1377	1.222	0.525	0.213	0.194
mean	0.703	0.542	0.201	0.2319	1.304	0.631	0.236	0.184
std deviation	0.318	0.325	0.065	0.0618	0.533	0.424	0.048	0.047

mf negative	IgG1	IgG1	IgG2	IgG2	IgG3	IgG3	IgG4	IgG4
	mmc-1	MBP	mmc-1	MBP	mmc-1	MBP	mmc-1	MBP
	0.652	0.580	0.495	0.1444	0.729	0.178	0.156	0.154
	0.353	0.353	0.107	0.2918	0.352	0.171	0.131	0.143
	0.209	0.345	0.190	0.3155	0.253	0.151	0.199	0.159
	0.955	0.465	0.295	0.3800	0.458	0.158	0.268	0.201
	0.685	0.460	0.357	0.3266	0.692	0.186	0.194	0.137
	0.429	0.346	0.124	0.2700	0.813	0.633	0.299	0.427
	0.709	0.389	0.150	0.2918	1.696	0.414	0.193	0.137
	1.496	0.472	0.143	0.1333	0.796	0.372	0.130	0.138
	1.277	0.607	0.199	0.1066	2.050	0.713	0.145	0.139
	0.801	0.532	0.187	0.2855	0.992	0.298	0.156	0.172
	1.551	1.289	0.162	0.2566	0.429	0.219	0.141	0.145
	0.469	0.678	0.329	0.2833	1.570	0.783	0.161	0.144
	0.752	0.457	0.352	0.2744	0.457	0.188	0.111	0.129
	1.032	0.300	0.228	0.2783	0.723	0.147	0.112	0.120
	0.558	0.317	0.149	0.1466	0.987	0.257	0.270	0.253
mean	0.795	0.506	0.231	0.2533	0.866	0.325	0.178	0.173
std deviation	0.401	0.244	0.111	0.0811	0.525	0.216	0.059	0.078

Euro Normal	IgG1	IgG1	IgG2	IgG2	IgG3	IgG3	IgG4	IgG4
	mmc-1	MBP	mmc-1	MBP	mmc-1	MBP	mmc-1	MBP
	0.303	0.390	0.169	0.126	0.130	0.115	0.127	0.131
	0.231	0.477	0.158	0.133	0.198	0.125	0.580	0.862
	0.556	0.485	0.174	0.121	0.191	0.131	0.913	0.563
	0.276	0.269	0.244	0.129	0.227	0.114	0.120	0.137
	0.844	0.579	0.188	0.145	0.256	0.119	0.122	0.130
	0.414	0.174	0.205	0.150	0.294	0.110	0.126	0.125
mean	0.437	0.396	0.190	0.134	0.216	0.119	0.331	0.325
std deviation	0.231	0.151	0.031	0.011	0.057	0.008	0.338	0.315

Figure 6.17 IgG subclass recognition of mmc-1 in human *B. malayi* infections.

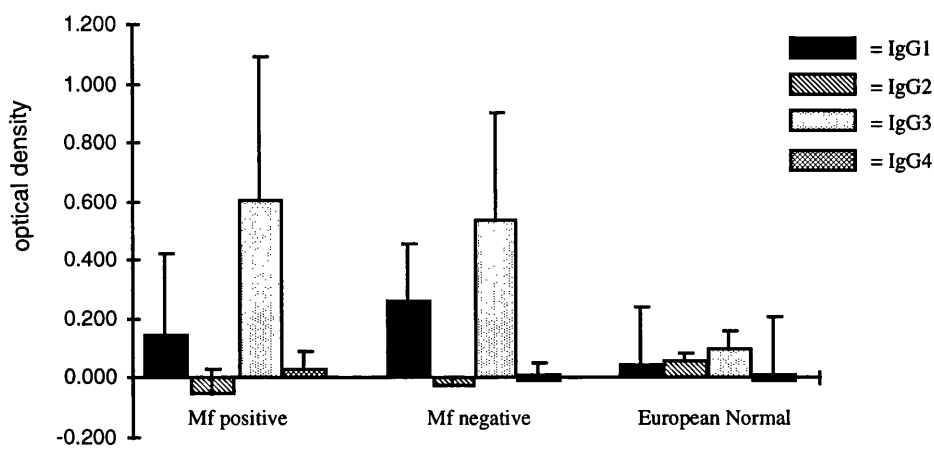


Figure 6.17

The profile of reaction of IgG subclasses to MMC-1 was tested in sera from mf + or mf - subjects or European normal control subjects. The data shown in Table 6.4 were plotted using OD readings for MBP-MMC-1 reactivity minus the MBP background reactivity of individual subjects. The graphs shows the mean of each group and standard deviations within each group.

Figure 6.18a shows proliferative responses of splenocytes from three groups of mice re-stimulated *in vitro* with 1 µg/ml of electro-eluted MMC-1 expressed as a stimulation index. No significant proliferation was observed in any group of animals. Figure 6.18b shows the proliferative response in the same groups of animals, when splenocytes were re-stimulated with mf antigen (10 µg/ml) or adult antigen (10 µg/ml). Cells from MMC-1 immunised mice failed to proliferate in response to either mf or adult antigen, whilst cells from mice immunised with soluble mf antigen responded well to re-stimulation with mf or adult antigen.

When the levels of cytokines produced by the cultured cells were measured by ELISA no significant amounts of IL-2, IL-4 IL-10 or IFN-γ were detected. The only cytokine that was produced in levels greater than the background was IL-5. Mice immunised with soluble mf antigen produced IL-5 in response to *in vitro* re-stimulation with either mf or adult antigen but not to MMC-1. In contrast the MMC-1 immunised animals re-stimulated with MMC-1 did produce significant amounts of IL-5 (Figure 6.19).

6.3 Discussion.

The experiments reported in this chapter aimed to define the role of *mmc-1* in the parasite life-cycle. As described previously, *mmc-1* appears to be a reasonably abundant mRNA exclusively expressed in the mf stage of the life-cycle. Unfortunately no clues as to the possible function could be gleaned from the analysis of the derived amino acid sequence. Consequently, the experiments described in the first part of this chapter focused on the basic immunochemical analysis of the protein. By SDS-PAGE and immunoblotting, the stage specificity of MMC-1 in the mf was

Figure 6.18a MMC-1 stimulated proliferative responses.

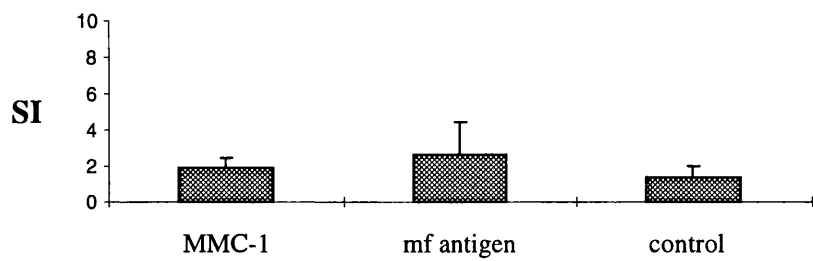


Figure 6.18b *B. pahangi* extract stimulated splenocyte proliferative responses.

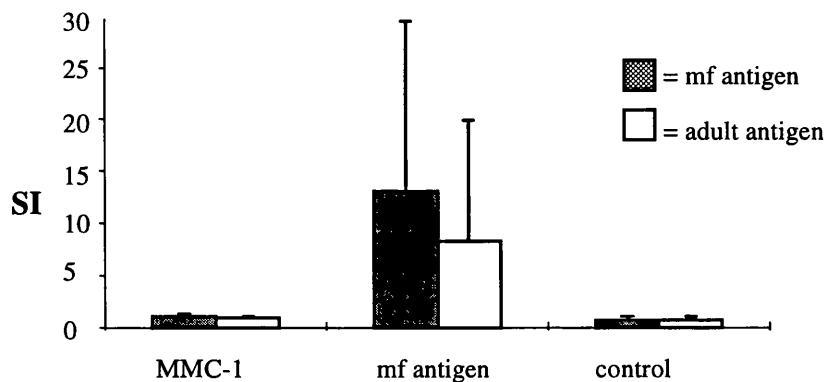


Figure 6.18a

Splenocytes purified from animals immunised on two occasions (day 0, day 14) with soluble mf antigen (50µg/mouse), MMC-1 (20 µg/mouse) or adjuvant only controls were re-stimulated *in vitro* by exposure to MMC-1 (1 µg/ml) or soluble mf antigen (10 µg/ml). Proliferation was measured by ³[H] thymidine incorporation. The results from 72 hour cultures and are plotted as stimulation indices, the mean proliferation of five animals per group are shown as are the standard deviations.

Figure 6.18b

Splenocytes purified from animals immunised as described above were stimulated with either soluble mf antigen or soluble adult antigen (both 10µg/ml). Proliferation was measured by ³[H] thymidine incorporation. The results from 72 hour cultures and are plotted as stimulation indices, the mean proliferation of five animals per group are shown as are the standard deviations.

Figure 6.19 Antigen specific production of IL-5.

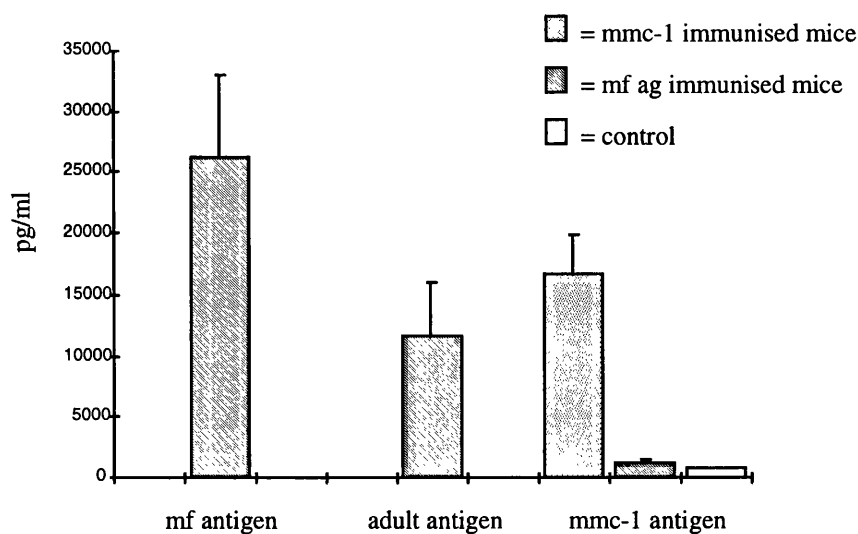


Figure 6.19
Splenocytes purified from five animals immunised on two occasions (day 0, day 14) with soluble mf antigen (50 $\mu\text{g}/\text{mouse}$), MMC-1 (20 $\mu\text{g}/\text{mouse}$) or adjuvant only (control) were tested for the production of IL-5 by capture ELISA after 72 hours in culture following re-stimulation with either soluble mf antigen (25 $\mu\text{g}/\text{ml}$), soluble adult antigen (10 $\mu\text{g}/\text{ml}$) or MMC-1 (0.5 $\mu\text{g}/\text{ml}$). The results are shown as mean IL-5 production in pg/ml from individual mice, and the standard deviation within each group.

confirmed. Two bands were recognised in the mf of approximately equal intensity. By comparison of mobility of molecular weight standards, these bands were estimated to have molecular weights of 8 kDa and 12.5 kDa. The molecular weight of the MMC-1 protein minus the signal peptide, predicted from the amino acid sequence was 7.4 kDa, consistent with the size of one of the proteins recognised by the anti-serum. The presence of the larger component may be due to modification of MMC-1 by glycosylation or post-translational modifications. The amino acid sequence of MMC-1 contains numerous potential phosphorylation sites although no potential glycosylation sites could be detected by computational analysis. However the presence of post-translational modification was not further investigated in the mature protein. Alternatively the larger of the two bands may represent a unrelated protein which shares a cross-reactive epitope with MMC-1. Lastly the 12.5 kDa band could represent a complex of MMC-1 with another protein, although this seems unlikely given the preparation of the sample (boiling 3 minutes in SDS-sample cocktail).

Next attempts were made to determine if MMC-1 was secreted from the parasite, by culturing mf *in vitro* and collecting and concentrating E/S products. E/S products of *Brugia* adult worms have been characterised using similar methods [23, 217, 229, 230], but there are few examples of detecting mf E/S products *in vitro*. Mf were cultured in medium containing serum or in serum-free medium. Although some components were apparent in medium containing serum, the chances are that these are serum proteins and not mf E/S products. In the serum-free medium, no E/S products could be detected. These results suggest that the MMC-1 protein is not excreted from the parasite. However this negative result may simply reflect the low level of excretion or the fact that the parasites were sub-optimally cultured. The silver

staining technique used will detect as little as 0.1 ng of protein in a single band [159] suggesting that little or no E/S products were produced under the conditions used. Kwan-Lim *et al* (1989) found that *B. malayi* adult parasites cultured *in vitro* took 3-4 days to adjust to the culture environment and that peak levels of excretion occurred on days 3 and 4 [217]. The adult parasites therefore seem to require a period of time to equilibrate with the conditions before maximal metabolism is restored. Parkhouse *et al* (1985)[231] reported that the labelling of mf with ³⁵S methionine, resulted in no detectable secreted proteins, although newly synthesised proteins synthesis could be detected in homogenized mf [231]. Therefore the mf may not produce E/S products under the conditions used. Why it should be so difficult to detect mf E/S products remains unknown. Mf are presumed to be metabolizing normally in the bloodstream and when mf are labelled with ³⁵S methionine *in vitro*, many proteins are labelled. It may be that very few proteins are secreted (this may correlate with the developmentally blocked state of the mf) or the proteins secreted may be of very low molecular weight and are may be lost during the concentration procedure (centrifugation with 5 kDa cut off concentration membrane). Alternatively, the secretion of MMC-1 may be dependent on a specific cue present at a later stage of development, such as when the parasite infects the mosquito host. Ibrahim *et al* (1992) showed that a number of antigens of *B. malayi* L3 parasites are synthesised in the late L2 stage but are only detectable at the parasite surface of the L3 [202]. Bianco *et al* (1990)[201] demonstrated that a 23 kDa antigen of *Onchocerca lienalis* was synthesised in the late L3 stage, but was released into culture only when the parasites were cultured at 37°C [201]. If the release/excretion of MMC-1 is controlled by environmental cues that were not present in the culture medium then

MMC-1 protein would not be detected in the E/S. As no system exists that allows *B. pahangi* mf to develop *in vitro* the possible secretion of MMC-1 as the mf develops would be difficult to investigate. It may have been possible to manipulate the culture conditions by changing culture temperature or media used to ascertain if this resulted in the release of MMC-1 into the media, however this was not attempted here.

The immunofluorescent localisation studies showed that MMC-1 was present throughout the mf and could not be localised to a particular tissue. The protein did not appear to be present on the mf sheath or cuticle (negative results with exsheathed mf). The internal localisation of an antigen does not preclude it from being involved in eliciting host immune reactions to the parasite as other antigens that are not predicted to be secreted or exposed have been found to immunologically important. For example, the heat shock cognate protein 70 (hsp70) is a dominant antigen in Brugian infections [232]. The protein is known to be cytoplasmic and only exposed after parasite death [233] as may be occurring with MMC-1.

By feeding mosquitoes on mf-infected blood containing MMC-1 IgG it was hoped that any possible role of MMC-1 in the infection of the mosquito host may be disrupted. These experiments were based on those used to determine the role of a filarial chitinase (discussed in Chapter Five) in the mosquito infection event. In the chitinase experiments, mosquitoes fed on blood containing the specific IgG did not support development of the parasites (J. Fuhrman personal communication). However, in the present experiments the inclusion of MMC-1 IgG or the control CDD IgG, had no effect on the development of the parasite to the L3 stage. However, to be effective in blocking the action of MMC-1, the anti-MMC-1 IgG must have access to an MMC-1 epitope. Therefore intervention by this method would only be

observed if the MMC-1 protein product is either secreted from the worm or is present on an exposed worm surface in the mosquito host. In the first experiment conducted the mosquitoes fed MMC-1 IgG had a much higher percentage of L3 in the abdomen compared to the other groups. The reasons for this result are not clear, as it is not known what determines the migration of the L3 in the mosquito. Certainly, MMC-1 did not appear to influence the migration of mf to the site of development or influence the development of the parasite in the thoracic muscles. The high percentage of L3 in the abdomen of mosquitoes fed MMC-1 IgG may be an artifact related to the heavy infection level in these mosquitoes. In the second experiment, the mosquitoes fed MMC-1 IgG contained significantly more L3 than the other two groups. As the infected blood for all three groups was prepared as a single sample before aliquoting into the groups it is difficult to explain the results. Further experiments in the mosquito vector are required to verify any conclusions.

The next experiments described in this chapter focused on the possible immunological role of MMC-1. The preliminary experiments showed that immunisation of BALB/c mice with MMC-1 antigen resulted in a specific IgG response (Figure 6.15). A very small reaction to MMC-1 was also seen when sera from BALB/c mice infected with mf was tested for MMC-1 specific IgG (Figure 6.16). The reasons why this reaction was minimal may relate to the internal location of the antigen and the short time course of infection (12 days). Perhaps if the BALB/c mice had been infected by a more immunogenic route of infection i.e. subcutaneously or the course of infection had been longer, the reaction to MMC-1 may have been greater.

The following experiments investigated the immune response of human subjects infected with *B. malayi*. The rationale supporting these experiments was that active infection with mf is associated with a range of immunological responses. For example in human subjects active infection with filarial worms is associated with specific IgG subclass responses. The predominant subclass in actively infected subjects is IgG4 where as much as 90% of total antifilarial antibodies will be of this subclass [26]. As the titre of IgG4 correlates with the numbers of mf in the bloodstream [106] it has been proposed that the presence of IgG4 may be a means to diagnose cryptic filarial infections where the numbers of circulating mf are very low [25, 106]. This is of particular importance for the diagnosis of *B. malayi* infections where no circulating antigen test exists. This predominance of IgG4 antibodies is not directed towards L3 antigens where the majority of IgG is of the IgG1 subclass [234]. Most experiments which measure IgG subclass responses to filarial antigens have used crude extracts of parasite antigens. It is therefore difficult to determine whether high levels of specific subclasses are directed against only a few or perhaps a single antigen. In order to determine if MMC-1 may be a target of filarial specific IgG4 antibodies, ELISAs were conducted with infected human sera. Interestingly no significant IgG4 response was detected to MMC-1, while both IgG1 and IgG3 were elevated in both mf positive and mf negative individuals. Previous studies have utilised this approach to study immune responses to individual antigens. Trenholme *et al* (1994)[109] showed that subclass specific immune responses to recombinant *O. volvulus* antigens (*Ov*MBP/10, *Ov*MBP/11 and *Ov*MBP/29) could be analysed in this way [109]. Later work by Bradley *et al* (1995) [235] showed that the recombinant antigen (*Ov*MBP20/11) was predominantly recognised by IgG1 and IgG4 subclasses,

and that significantly higher IgG1 was observed in serum from actively infected patients compared to that of putative immune patients [235]. In contrast the *O. volvulus* collagen (*Ov-col-1*) has been shown to be preferentially recognised by IgG3 of putatively immune individuals [236]. Similar studies have also been conducted using recombinant antigens of *Brugia spp.* Yazdanbakhsh *et al* (1995) [233] showed that the antibody responses to two recombinant antigens of *Brugia spp* were again correlated with clinical status [233]. Elephantiasis patients showed a greater reactivity to both *BpL-4* (one repeat of the secreted polypeptide gp15/400) and *Bpa-26* (C-terminal portion of hsp70, a cytoplasmic protein) than mf- or mf+ patients. Also significantly greater IgG1 reactivity was seen to both antigens in serum of elephantiasis patients compared to mf + or mf - patients.

Infection with filarial parasites can result in a range of clinical manifestations from asymptomatic microfilaraemias to chronic pathology. Patients with circulating mf are often hyporesponsive to parasite antigens [13, 106], whilst individuals that show pathological symptoms tend to have a reduction in mf numbers or no mf present in the circulating blood stream and heightened immune responses. This has led to the proposal that the breakdown of tolerance may result in inflammatory responses and in the induction of pathology [106]. As IgG3 antibodies are responsible for type III hyper-responsiveness due to the human Fc receptor on monocytes, macrophages and granulocytes having a higher affinity for IgG3 [237], reaction to IgG3 stimulating antigens may be a factor involved in this process.

High titres of IgG1 and IgG3 and low titres of IgG4 have also been associated with chronic pathology in patients infected with *B. malayi* [26, 106, 238], *O. volvulus* [236] and *W. bancrofti* [94, 239]. It has also been reported that two major parasite

antigens (72 kDa and 12 kDa) of *O. volvulus* are recognised solely by IgG3 in SOWDA (severe hyper-reactive dermatitis) patients [240].

The data obtained from the cytokine ELISAs showed that immunisation of mice and re-stimulation of splenocytes with MMC-1 resulted in the production of significant amounts of IL-5. IL-5 is a developmental regulator and survival factor for eosinophils and results in the increase of eosinophils in the circulation. IL-5 stimulates the differentiation of bone marrow CD34⁺ precursors to develop into eosinophils then promotes the mobilization of the cells from the bone marrow. It has also been shown to act as a survival factor by inhibition of eosinophil apoptosis [241, 242].

By eliciting IgG3 antibodies and elevating IL-5, could MMC-1 be involved in promoting a pathological state in *Brugia* infection by induction of eosinophilia? Helminth infections are known to stimulate increased numbers of mast cells and eosinophils and the role of eosinophils in *in vitro* killing of *B. malayi* infective larvae [243], *S. mansoni* schistosomula [244, 245] and *H. contortus* L3 [246] is well documented. It has also been shown that the cytotoxic effects of eosinophils is mediated by the IgG subclasses IgG1, 2 and 3. Khalife *et al* (1989)[244] showed that although IgG1, 2, 3 and 4 all bind to the surface of schistosomula of *S. mansoni* only IgG1 2 and 3 antibodies could induce cytotoxicity of activated eosinophils [244].

One of the most severe pathologies to occur in filarial infections is TPE, which is thought to reflect an allergic response to mf in the lung [15]. In this condition mf are very rarely seen in blood but treatment with DEC results in relief of symptoms. The wheezing cough and chest pain associated with TPE is thought to result from damage to the lung from degranulation of eosinophils in response to dead

and degenerating mf present in the lung. Hall *et al* (1998) [247] showed that following intravenous infection of C57BL/6 mice with mf, the parasites were detectable in the lung after 1 day and eosinophils were observed on the surface of the mf [247]. Resulting damage of the lung was associated with the mf after staining for major basic protein. A hallmark of TPE is the detection of eosinophils in the bronchoalveolar lavage (BAL). When mice are sensitised by immunisation with dead mf then challenged with live mf 3.8 % of the cells present in the BAL were eosinophils at day one post challenge. This figure rose steeply to 40 % at day 4 and 84 % by day 10 post challenge [247]. The eosinophilia seen is under the control of IL-5 as IL-5^{-/-} knockout mice do not exhibit eosinophilia in the airways [247]. Also in other studies blocking IL-5 by antibodies inhibits eosinophilia [248, 249].

Lobos *et al* (1992) showed that a *B. malayi* allergen Bm23-25 (composed of two antigens of 23 and 25 kDa) which is predominately expressed by the mf stage is detected solely by patients suffering TPE [250]. Bm23-25 was shown to be the major component recognised by TPE patient sera and was found to be reactive to the BAL fluid of TPE sufferers. It is therefore possible that only a small number of antigens may be responsible for activating the immune responses leading to the pathology associated with TPE.

Although the possible role of MMC-1 in the induction of eosinophilia was not tested it is an intriguing hypothesis that antigens such as MMC-1 may induce IL-5 production and a resulting eosinophilic response. The predominance of IgG3 antibodies detected in the human serum may also be involved in the degranulation of activated eosinophils. This hypothesis is speculative and further experiments to determine the role of MMC-1 in this process could include determining if mice

exposed to MMC-1 would produce an eosinophilic response; determining if the cellular infiltrate seen following peritoneal infection with mf (personal observation) could be stimulated by injection of MMC-1 alone; or determining if MMC-1 could activate stimulated eosinophils to degranulate. If MMC-1 has such a role in *Brugia* infections then it might be predicted that the mf negative and mf positive groups from the IgG subclass ELISA experiments would show different levels of MMC-1 reactive IgG3 antibodies, perhaps correlating to the amount of mf antigen that the immune system was exposed to. Although it has been shown that following the clearance of mf by DEC treatment, the levels of IgG1 and IgG4 drop as would be expected, whilst the levels of IgG3 did not consistently alter 12 months after treatment [251].

In summary the experiments carried out in this chapter in an attempt to elucidate the function of MMC-1 were inconclusive. However several interesting lines of evidence suggest that MMC-1 may be an important molecule for further analysis, particularly with regard to the immune response elicited.

CHAPTER SEVEN

7.1 Conclusions.

The screening approach described here isolated a number of genes that are differentially expressed in the mf of *B. pahangi*. Of the nine clones isolated, five were up-regulated in the mammalian-derived mf and four in the vector-derived mf. Six of the clones were homologous to cDNAs previously characterised from other species. The remaining three clones represented novel cDNAs without a proposed function. It was these novel genes that were of particular interest.

The two cDNAs, *vmc-1* and *vmc-2* which were up-regulated in the vector-derived mf, were of interest as little is known about the biology of *Brugia* when in the mosquito vector. The gene *vmc-1* is completely novel and has no homology to any characterised gene or sequenced EST, although the small size of the transcript which may account for this, meant that no further analysis was conducted. A *vmc-2* homologue has not yet been isolated by the *B. malayi* EST project, suggesting that it may be expressed at low levels in the libraries studied to date. The sequence does show some homology to a potential glycerophosphoryl diester phosphodiesterase (glp) of *C. elegans*, although verification of *vmc-2* as a glp was not conducted in this study. The expression level of *vmc-2* mRNA increases throughout development in the mosquito vector, but is barely detectable after 24 hours in the mammal. Thus *vmc-2* is potentially important in the preparation of the parasite for infection of the mammalian host. It would be interesting to determine if the VMC-2 protein may have a role in the infection event as has been proposed for a 23 kDa antigen of *Onchocerca* spp [201]. Like *vmc-2* the 23 kDa *Onchocerca* antigen was found to increase in abundance whilst the parasite matured from L2 to L3 in the vector host. Metabolic labelling of parasite proteins whilst in the blackfly vector and during

subsequent *in vitro* culture showed that the protein was first detectable in the L2 stage and was highly abundant in the L3 stage. Interestingly the abundance of the protein was shown to reduce following 24 hours culture at 37°C. It would be interesting to determine the levels of VMC-2 protein in L3 of *B. pahangi* isolated from the mosquito compared to those cultured in the mammalian-like conditions.

The majority of the work focused on *mmc-1* because this was the most abundant clone isolated and the mRNA was exclusive to the mf stage. The specialisation of the L1 of *Brugia* is evident in its anatomy, physiology and behaviour, therefore the specific temporal expression of the gene suggested it may play a role in mf development or mf specific processes.

Preliminary analysis of the *mmc-1* cDNA and translated protein sequences revealed that *mmc-1* was a novel gene with no known homologues other than to ESTs of *B. malayi*. This was later confirmed by the lack of hybridisation to DNA of other filarial nematodes. It was unfortunate that sufficient genomic DNA was not available from *W. bancrofti* to conduct this experiment. *W. bancrofti* shows many similar traits to *B. pahangi* (blood dwelling, possession of microfilarial sheath, microfilarial periodicity) and may have been the most obvious candidate for a homologue outwith *Brugia*. The PCR method used on a *W. bancrofti* cDNA library did not result in the amplification of a homologous gene. This protocol was only attempted using gene-specific primers and perhaps a degenerate PCR approach may have provided a different conclusion. The predicted amino acid sequence was analysed to determine any potential protein family domains or signatures by various computational methods [124, 252, 253]. However these techniques did not provide any information that would allow the prediction and testing of MMC-1 function. In

an attempt to resolve possible functions of *mmc-1*, experiments using MMC-1 recombinant protein or MMC-1 anti-serum were conducted.

Using immunofluorescence MMC-1 appeared to be localised throughout the body of whole *B. pahangi* mf, but only after removal of the microfilarial sheath and permeabilisation with collagenase and β -mercaptoethanol. This demonstrated that the MMC-1 protein was not present at the surface of the sheath or in the cuticle but was likely to be cytoplasmic. Attempts to localise MMC-1 at the ultrastructural level are ongoing.

As the mf stage has been shown to be involved in the modulation of host immune responses it was possible that mf-specific antigens, such as MMC-1 may be involved in this phenomenon. ELISA experiments showed that the immunisation of mice with a MMC-1 fusion protein resulted in the production of significant amounts of MMC-1 specific antibodies. MMC-1 was therefore immunogenic in isolation, however when a similar experiment was conducted using animals infected with either mf or L3 of *B. pahangi* only low levels of anti-MMC-1 IgG was detected in mf infected mice. This may be intuitive as MMC-1 was not localised to the surface of the parasite and may therefore only be presented to the immune system upon death of the mf. Experiments utilising sera from *B. malayi* infected individuals showed that the predominant MMC-1 reactive IgG subclasses were IgG1 and IgG3. In addition splenocytes from MMC-1 immunised animals, restimulated with MMC-1, were shown to produce significant amounts of IL-5. Together these findings are very interesting, as high titres of IgG1 and IgG3 have been shown to correlate with pathology in filarial infections [106, 239]. Also IL-5 is a growth factor for eosinophils that are present in high numbers in the pathological state of TPE [241,

247, 249]. As the pathology observed in TPE is thought to be associated with an allergic reaction to mf in the lung, [15, 247] it was hypothesised that MMC-1 may be an antigen of importance in these allergic reactions, perhaps by stimulating IL-5 production. Alternatively eosinophils may be stimulated to degranulate in association with MMC-1 IgG1 and IgG3 antibodies as has been described in the eosinophil-mediated killing of *S. mansoni* schistosomula which is dependent on human IgG subclasses 1, 2 and 3 [244].

7.2 Further experiments.

The experiments reported in this thesis did not provide evidence as to how *mmc-1* expression is regulated. It was however possible to rule out temperature, and the presence of glucose or serum as stimulation factors. Little is known about the control of gene expression in helminth parasites. This is partly because upstream regions have not been cloned and partly because of the lack of an *in vitro* system constrains experimentation. Analysis of the activity of promoters of helminth genes is in its infancy and has mostly relied on the use of reporter gene constructs. For these experiments constructs are made in which a detectable reporter gene is placed under the control of the promoter of interest and the construct is transfected into a eukaryotic cell line. This has been successful in the analysis of *S. mansoni hsp70* [254] and *O. volvulus* superoxide dismutase [255]. This approach has also been used in this laboratory by transfection of mammalian COS-7 cells with a *hsp90* promoter::chloramphenicol acetyl transferase (CAT) reporter construct (A. Coxcroft, unpublished). It is also possible to determine if the promoter of a parasitic gene can direct expression of *C. elegans* genes although this has only been conducted with

parasite genes with a *C. elegans* homologue [136]. It may be possible to attempt this type of experiment using a parasite specific gene, however the pattern of expression both temporal and spatial may not directly correlate to expression in the parasite species.

The hypothesis that MMC-1 is a potential allergen in conditions such as TPE is based on preliminary data that would need to be repeated in greater detail. It has been shown that immunisation of mice with heat killed mf of *B. malayi* and subsequent challenge with live mf produces both pulmonary and peripheral eosinophilia [247]. To determine if MMC-1 is involved in this process, similar experiments could be conducted with mice sensitised by MMC-1 immunisation and then challenge with mf. By comparing eosinophil recruitment into the peritoneal cavity of MMC-1 primed mice with that in mice immunised with an irrelevant antigen it would be possible to further dissect the role of MMC-1. Alternatively it has been shown that a *B. malayi* mf allergen (Bm2325) was recognised by serum from TPE patients [250]. This would be a relatively simple experiment to conduct by western blot of recombinant MMC-1. Interestingly the Bm2325 antigen has been described as a homologue of the precursor of gamma-glutamyl transpeptidase, an enzyme present in the host epithelial cells. It has been proposed that molecular mimicry between host and parasite proteins may lead to the pathology seen. However this is not likely to be the case with MMC-1 as it does not show homology to any characterised proteins.

7.3 Future prospects.

Genes which demonstrate a specific expression pattern during the life-cycle of a parasite are of interest as the resulting proteins may be involved in specific metabolic pathways or developmental processes that would otherwise remain undetected. As discussed in Chapter One the challenge for molecular parasitologists is to assign function to the many genes that are isolated by genomic approaches. The Filarial Genome Project has isolated over 27, 000 ESTs of which approximately 40 % are currently assigned as novel genes [123]. As the Filarial Genome Project progresses and more data are generated, future work is likely to incorporate procedures for genome wide analysis of expression utilising methods such as DNA chip technologies. These methods utilise short nucleic acid sequences that are attached to a solid support in an ordered fashion to analyse the genes expressed under any testable condition [256, 257]. By the use of two colour labelling of different populations of cDNAs, the genome-wide expression of genes can be assessed [258]. These experiments provide not only information on which genes are expressed at any time point but can also provide information that can be used to infer a possible function. Unrelated genes that are expressed in the same manner following different treatments e.g. temperature shock or drug treatment may be controlled by the same process and be part of a pathway that allows putative functions to be assigned and tested.

In model organisms such as *C. elegans* the generation of animals with mutations in a gene of interest allows the placing of gene products in an ordered pathway [259, 260]. These techniques are not applicable to the study of *B. pahangi* genes due to the complexity of the life-cycle. The alternative approach of double

stranded RNA interference (dsRNAi), [261] involves the interference of an endogenous gene by the introduction of specific double stranded RNA. The technique has been widely used in *C. elegans* but has also been used in *Trypanosoma brucei* [262], *Drosophila* cell lines [263] and mice [264]. It has recently been proposed that RNAi directs cleavage of the gene specific RNA, which results in the loss of expression [265]. The use of this technique in *C. elegans* is a powerful tool to determine the function of specific genes, and how the loss of gene function affects the nematode. For genes such as *mmc-1* that are novel with no homology to genes of model organisms, the lack of such tools to determine gene function can be a frustrating problem. *mmc-1* is good candidate for attempts to conduct dsRNAi in a filarial nematode due to its abundance and stage specificity. As there is no *in vitro* system that allows the culture of *B. pahangi* through a complete life cycle the only stage that could realistically be studied following introduction of RNA into adult females would be the mf. Although traditionally micro-injection techniques have been used to introduce the dsRNA, the technique has been shown to be effective when *C. elegans* are fed on bacteria expressing the dsRNA [266] or by soaking the worms in a solution of dsRNA [267]. As adult *B. pahangi in vitro* have been shown to absorb substances across the cuticle [220] this may allow the incorporation of dsRNA into adult females. The major problem with this type of experiment would be determining the effect on the progeny. If the function or specific localisation of the protein were known then potential phenotypic changes may be deduced. If a behavioural mutation resulted then it would be more difficult to detect this in a filarial worm.

In conclusion *mmc-1* is a potentially significant gene that as yet has an uncharacterised function. Preliminary functional experimentation suggested that *mmc-1* may have a role in the immune reactions seen in filarial infections. There are a number of possible hypothesis that could be readily tested to elucidate the function of this important gene.

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